

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁵ : C08G 63/48, 63/91, C08H 1/00 A61K 37/12	A1	(11) International Publication Number: WO 94/01483 (43) International Publication Date: 20 January 1994 (20.01.94)																		
(21) International Application Number: PCT/US93/06292 (22) International Filing Date: 1 July 1993 (01.07.93) (30) Priority data: <table border="0"><tr><td>07/907,518</td><td>2 July 1992 (02.07.92)</td><td>US</td></tr><tr><td>07/922,541</td><td>30 July 1992 (30.07.92)</td><td>US</td></tr><tr><td>07/984,933</td><td>2 December 1992 (02.12.92)</td><td>US</td></tr><tr><td>07/984,197</td><td>2 December 1992 (02.12.92)</td><td>US</td></tr><tr><td>07/985,680</td><td>2 December 1992 (02.12.92)</td><td>US</td></tr><tr><td>08/025,032</td><td>2 March 1993 (02.03.93)</td><td>US</td></tr></table> (71) Applicant: COLLAGEN CORPORATION [US/US]; 2500 Faber Place, Palo Alto, CA 94303 (US). (72) Inventors: RHEE, Woonza ; 3860 Timlott Court, Palo Alto, CA 94306 (US). WALLACE, Donald, G. ; 290 Ringwood Avenue, Menlo Park, CA 94025 (US). MICHAELS, Alan, S. ; 316 Beacon Street, Boston, MA 02116 (US). BURNS, Ramon, A., Jr. ; 563 Becado Place, Fremont, CA 94539 (US). FRIES, Louis ; 1684 Clay Drive, Los Altos, CA 94024 (US). DELUSTRO, Frank ; 2517 DeKoven Avenue, Belmont, CA 94002 (US). BENTZ, Hanne ; 36125 Toulouse Street, Newark, CA 94560 (US). MCCULLOUGH, Kimberly ; 29858 Clearbrook Circle, #124, Hayward, CA 94544 (US). DAMANI, Ramesh ; 1600 Villa Street, #223, Mountain View, CA 94041 (US). BERG, Richard, A. ; 1055 Manet Drive, #42, Sunnyvale, CA 94087 (US).		07/907,518	2 July 1992 (02.07.92)	US	07/922,541	30 July 1992 (30.07.92)	US	07/984,933	2 December 1992 (02.12.92)	US	07/984,197	2 December 1992 (02.12.92)	US	07/985,680	2 December 1992 (02.12.92)	US	08/025,032	2 March 1993 (02.03.93)	US	(74) Agents: BOZICEVIC, Karl et al.; Morrison & Foerster, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US). (81) Designated States: AU, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
07/907,518	2 July 1992 (02.07.92)	US																		
07/922,541	30 July 1992 (30.07.92)	US																		
07/984,933	2 December 1992 (02.12.92)	US																		
07/984,197	2 December 1992 (02.12.92)	US																		
07/985,680	2 December 1992 (02.12.92)	US																		
08/025,032	2 March 1993 (02.03.93)	US																		
(54) Title: BIOCOMPATIBLE POLYMER CONJUGATES (57) Abstract Pharmaceutically acceptable, non-immunogenic conjugates are formed by covalently binding a biologically inactive, natural polymer or derivative thereof to pharmaceutically pure, synthetic hydrophilic polymers via specific types of chemical bonds. The biocompatible conjugates can be used for soft tissue augmentation and for coating or forming various articles. The synthetic hydrophilic polymer may be polyethylene glycol and derivatives thereof having a weight average molecular weight over a range of from about 100 to about 100,000. The compositions may include other components such as liquid, pharmaceutically acceptable carriers to form injectable formulations, and/or biologically active proteins such as growth factors or cytokines. The conjugates generally contain large amounts of water when formed and can be dehydrated to form a solid object which can subsequently be ground into particles and suspended in a non-aqueous fluid for injection into a mammal to provide the soft tissue augmentation.																				

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

-1-

5 BIOCOMPATIBLE POLYMER CONJUGATESTechnical Field

 This invention relates to biocompatible conjugates
formed by covalently binding two or more polymers
10 together and, specifically, to conjugates formed by
binding a naturally occurring polymer or derivative
thereof to a synthetic hydrophilic polymer, such as
polyethylene glycol (PEG), and to compositions,
components, and implants comprised of such conjugates.

15

Background of the Invention

 A number of naturally occurring but biologically
inert polymers are known. Examples of such include
collagen and various glycosaminoglycans such as
20 hyaluronic acid, chondroitin sulfates, chitin, and
heparin. Derivatives of these polymers have also been
produced and certain derivatives have been formulated for
medical use. A number of synthetic biologically inert
polymers are also known, e.g., polyethylene glycol (PEG).
25 Various combinations of natural and synthetic polymers
give a wide range of characteristics for use in a variety
of medical applications. We have now invented new and
useful conjugates combining the characteristics of
natural and synthetic polymers and applied these
30 conjugates to medical formulations, components, and
implants which specifically benefit from these
characteristics.

35

-2-

Summary of the Invention

Conjugates are formed by covalently binding insoluble, naturally occurring, biologically inert polymers and derivatives thereof to synthetic, hydrophilic polymers such as polyethylene glycol (PEG). The naturally-occurring polymers and derivatives thereof include polysaccharides such as hyaluronic acid, proteoglycans such as chondroitin sulfate A (4-sulfate), chondroitin sulfate C (6-sulfate), and dermatan sulfate (chondroitin sulfate B); chitin; heparin and heparin sulfate; dextrans such as cyclodextran, hydroxyethyl cellulose, cellulose ether, and starch; lipids (esters of fatty acids with trihydroxyl alcohol glycerol) such as triglycerides, phospholipids, and the like. The synthetic hydrophilic polymer is preferably polyethylene glycol and derivatives thereof having a weight average molecular weight in the range of from about 100 to about 100,000 preferably about 1,500 to 20,000. Compositions and components may be formulated using the conjugates and other components such as pharmaceutically acceptable fluid carriers to form injectable formulations, and/or biologically active proteins such as cytokines and growth factors. The biocompatible conjugates of the invention generally contain large amounts of water when formed and can be dehydrated to form a relatively solid object which will expand in size five-fold or more upon rehydration. Implants can be coated with conjugate formulation, and articles such as tubes and strings can be constructed using specific conjugates and formulations in order to obtain the particular desired characteristics.

The biocompatible conjugates of the invention are applied and used in a variety of medical and pharmaceutical applications. The most basic embodiment includes the biocompatible conjugates and pharmaceutical compositions formulated using these conjugates, which

-3-

compositions include pharmaceutically acceptable fluid carriers of varying types and amounts. When forming the conjugates, various natural polymers are covalently bound to synthetic polymers such as polyethylene glycol. The specific polymers are chosen based on the end use and characteristics desired. In addition to selecting the polymers to be bound, different types of covalent bonds can be used, including ester, ether and urethane linkages.

One of the most important uses for certain conjugates and compositions of the invention is in methods of augmenting soft tissue. The compositions are formulated in a flowable form and injected into patients, such as into facial areas, to provide for soft tissue augmentation. The method can be varied so that the reaction between the naturally occurring polymer and the synthetic polymer occurs in situ. The conjugates can be dehydrated and then ground into particles, suspended in an inert nonaqueous carrier, and injected into a patient. After injection, the carrier will be removed by natural physiological conditions and the particles will rehydrate and swell to their original size. Strings formed from conjugates can also be injected to obtain soft tissue augmentation.

The conjugates and conjugate compositions of the invention can be combined with cytokines or growth factors to promote tissue growth, and/or further combined with particles, fibers or other materials to increase the structural integrity of the compositions so that they can also be used in the augmentation of hard tissue, such as bone and cartilage. Other uses for conjugates include coatings for various medical devices to be incorporated within the body, including catheters, bone implants, and platinum wires to treat aneurysms. The conjugates may also be formulated into various ophthalmic devices, such

- 4 -

as lenticules or corneal shields. Conjugate formulations may also be extruded, molded, and/or formed into shapes such as strings and tubes which have medical uses such as in sutures and blood vessel or nerve repair.

5 A primary object of the invention is to provide biocompatible conjugates formed by covalently binding polymers such as polyethylene glycol to non-immunogenic forms of naturally occurring, insoluble, biologically inert polymers and derivatives thereof.

10 Another object of the invention is to provide the conjugates with different types of bonds and to provide compositions containing conjugates in pharmaceutically acceptable fluid carriers suitable for injection.

15 Another object of the invention is to provide a composition for tissue augmentation produced by forming the conjugates, dehydrating the conjugates to form a solid, grinding the solid into particles, and suspending the particles in a non-aqueous fluid carrier for injection to the site of augmentation, at which time the
20 particles will rehydrate and expand in size about five-fold.

 Another object of the invention is to provide objects such as implants which have biocompatible conjugate formulations coated on their surfaces.

25 Yet another object of the invention is to provide strings made of the conjugate materials.

 Still another object of the invention is to provide tubes made of the conjugate materials.

30 An important advantage of the present invention comprising the biocompatible conjugates is that particular types of covalent bonds, such as ether linkages, can be used to provide a high degree of stability over long periods of time under physiological conditions.

35

-5-

A feature of the invention is that the conjugates can be formed using a variety of natural and synthetic polymers, each occurring in a range of molecular weights in order to adjust the physical and chemical characteristics of the resulting composition.

Another advantage of the present invention is that the biocompatible conjugates have superior handling characteristics as compared with conventional collagen compositions.

Another advantage of the present invention is that the biocompatible conjugate compositions generate a decreased immune reaction as compared with conventional compositions.

Another feature of the present invention is that the biocompatible conjugate compositions have improved moldability, malleability, and elasticity as compared with conventional compositions.

Other features of the present invention include the ability to formulate the compositions and conjugates in combination with pharmaceutically active proteins such as cytokines and growth factors in order to improve the activity and available half-life of such cytokines or growth factors under physiological conditions.

Another advantage of the present invention is that an ether linkage may be used to connect the natural and synthetic polymers which bond is resistant to breakage due to hydrolysis.

These and other objects, advantages, and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the structure, synthesis, and usage of the biocompatible conjugates as more fully set forth below, reference being made to the specific examples and formulations forming a part hereof.

35

-6-

Brief Description of the Drawings

Figure 1 is a bar graph showing the results of swellability testing for different strings;

5 Figures 2, 3, 4 and 5 are each bar graphs showing the results of testing physical characteristics of different strings;

Figures 6 and 7 each show comparisons of the DSC measurements of collagen containing compositions;

10 Figure 8 shows the reaction scheme for reacting a carboxyl group of hyaluronic acid with PEG-hydrazine;

Figure 9 shows a reaction scheme wherein an acetyl group of hyaluronic acid is reacted with succinimydyl-PEG;

15 Figure 10 shows the structures of chondroitin sulfate A, chondroitin sulfate C and dermatan sulfate (chondroitin sulfate B);

Figure 11 is a reaction scheme showing a nucleophilic substitution reaction of an ester with PEG;

20 Figure 12 shows the reaction scheme of reacting polyethylene with a multifunctional activated form of E-PEG;

Figure 13 shows a reaction scheme reacting polyethylene with a multifunctional activated form of S-PEG;

25 Figure 14 is a table showing the results of measurements of strings produced in accordance with Example 13;

Figure 15 is a table showing physical data relating to strings comprised of collagen and collagen conjugates;

30 Figure 16 is a table showing data obtained from measurements of materials produced in accordance with Example 15; and

Figure 17 is a table showing the results of measurements obtained on materials produced in accordance with Example 18.

35

-7-

Detailed Description of Preferred Embodiments of the
Invention

Before the present biocompatible conjugates and processes for making and using such are described, it is
5 to be understood that this invention is not limited to the particular conjugates, processes, and methods described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is
10 not intended to be limiting, as the scope of the present invention will be limited only by the appended claims.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context
15 clearly dictates otherwise. Thus, for example, reference to "a naturally occurring polymer" includes mixtures of such polymers, reference to "an attaching group or a linking group" includes one or more different types of groups known by those skilled in the art, or those which
20 are capable of forming a covalent bond, and reference to "the synthetic polymer" includes mixtures of different types of polymers such as polyethylene glycol (PEG) and so forth.

Unless defined otherwise, all technical and
25 scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein may be useful in the practice or testing of the
30 present invention, preferred methods and materials are described below. All publications mentioned herein are incorporated herein by reference. Further, specific terminology of particular importance to the description of the present invention is defined below.

35

-8-

A. Definitions

The term "collagen" as used herein refers to all forms of collagen, including telopeptide-containing collagen, and atelopeptide collagen which has been processed or modified. The collagen may be of human or animal origin and may be produced by recombinant techniques. Forms included are the various types, i.e., Type I, II, III, fibrillar, non-fibrillar, etc. Collagen is a material which is the major protein component of bone, cartilage, skin, and connective tissue in animals. Collagen in its native form is typically a rigid, rod-shaped molecule approximately 300 nm long and 1.5 nm in diameter. It is composed of three collagen polypeptides which form a tight triple helix. The collagen polypeptides are characterized by a long midsection having the repeating sequence -Gly-X-Y-, where X and Y are often proline or hydroxyproline, bounded at each end by the "telopeptide" regions, which constitute less than about 5% of the molecule. The telopeptide regions of the collagen chains are typically responsible for the naturally occurring crosslinking between chains, as well as for the immunogenicity of the protein. Collagen occurs in several "types" having differing physical properties. The most abundant types are Types I-III. The present disclosure includes these and other known types of collagen including natural collagen and collagen which is processed or modified, i.e., various collagen derivatives. Collagen is typically isolated from natural sources, such as bovine hide, cartilage, or bones. Bones are usually dried, defatted, crushed, and demineralized to extract collagen, while hide and cartilage are usually minced and digested with proteolytic enzymes (other than collagenase). As collagen is resistant to most proteolytic enzymes, this procedure conveniently serves

35

-9-

to remove most of the contaminating protein found with collagen.

The term "dehydrated" refers to compositions that have been air dried or lyophilized to remove
5 substantially all unbound water.

The terms "naturally occurring polymers" and "natural polymer" as used herein refer to biologically inert, insoluble, naturally occurring, biocompatible polymers and derivatives thereof. Examples of natural
10 polymers include polysaccharides such as hyaluronic acid; proteoglycans such as chondroitin sulfate A (4-sulfate), chondroitin sulfate C (6-sulfate), and dermatan sulfate (chondroitin sulfate B); dextrans such as cyclodextrin, hydroxyethyl cellulose, cellulose ether, and starch;
15 lipids (esters of fatty acids with trihydroxyl alcohol glycerol) such as triglycerides, phospholipids, and mixtures and derivatives thereof. The term is intended to specifically exclude biologically active natural polymers such as DNA, RNA, and proteins.

20 The terms "biologically inert conjugates", "biocompatible conjugates", and "biologically inert biocompatible conjugates" are used interchangeably herein. The terms refer to biologically inert, insoluble, biocompatible conjugates of the present
25 invention wherein a natural polymer is covalently bound to the synthetic hydrophilic polymer. The conjugates have some characteristics similar to the natural polymers in that they are biologically inert, insoluble, nontoxic and do not generate a significant immune reaction when
30 incorporated into a living being.

The term "synthetic hydrophilic polymer" as used herein refers to a synthetic polymer having an average molecular weight and composition which renders the polymer essentially hydrophilic, but not completely
35 water-soluble. Preferred polymers are highly purified

-10-

such that the polymer is pharmaceutically pure. Most hydrophilic polymers can be rendered water-soluble by incorporating a sufficient number of oxygen (or less frequently, nitrogen) atoms available for forming

5 hydrogen bonds in aqueous solutions. Preferred polymers are hydrophilic, but not necessarily soluble. Hydrophilic polymers used herein include polyethylene glycol (PEG), polyoxyethylene, polymethylene glycol, polytrimethylene glycols, polyvinylpyrrolidones, and

10 derivatives thereof, with PEG being particularly preferred. The polymers can be linear or multiply branched and will not be substantially crosslinked. Other suitable polymers include polyoxyethylene-polyoxypropylene block polymers and copolymers.

15 Polyoxyethylene-polyoxypropylene block polymers having an ethylene diamine nucleus (and thus having four ends) are also available and may be used in the practice of the invention. Naturally occurring polymers such as

20 proteins, starch, cellulose, heparin, and the like are expressly excluded from the scope of this definition. All suitable polymers will be non-toxic, non-inflammatory, and non-immunogenic when administered subcutaneously, and will preferably be essentially

25 nondegradable in vivo over a period of at least several months. The hydrophilic polymer may increase the hydrophilicity of the natural polymer, but does not render it water-soluble. Presently preferred hydrophilic polymers are mono-, di-, and multifunctionally activated polyethylene glycols (PEG). Monofunctional PEG has only

30 one reactive hydroxy group, while difunctional PEG has reactive groups at each end of the molecule. Monofunctional PEG preferably has a weight average molecular weight between about 100 and about 15,000, more preferably between about 200 and about 8,000, and most

35 preferably about 4,000. Difunctional PEG preferably has

-11-

a weight average molecular weight of about 400 to about 100,000, more preferably about 3,000 to about 20,000. Multifunctional PEG preferably has an average molecular weight between about 3,000 and 100,000.

5 The term "multifunctional" is used herein to refer to synthetic polymers having two or more reactive groups per molecule and, as such, encompasses the term "difunctional."

10 The terms "monofunctional", "difunctional", and "multifunctional" are used interchangeably herein with the terms "monofunctionally activated", "difunctionally activated", and "multifunctionally activated", respectively.

15 PEG can be rendered monofunctional by forming an alkylene ether group at one end. The alkylene ether group may be any suitable alkoxy radical having 1-6 carbon atoms, for example, methoxy, ethoxy, propoxy, 2-propoxy, butoxy, hexyloxy, and the like. Methoxy is presently preferred. Difunctionally activated PEG is
20 provided by allowing a reactive hydroxy group at each end of the linear molecule. The reactive groups are preferably at the ends of the polymer, but may be provided along the length thereof. Multifunctionally activated molecules are capable of crosslinking the
25 compositions of the invention, and may be used to attach cytokines or growth factors to natural polymers. In connection with the synthetic polymers, certain abbreviations are used as follows:

monomethoxypolyethylene glycol (mPEG); difunctional PEG
30 Succinimydyl Glutarate (SG-PEG); difunctional PEG Succinimydyl (S-PEG); difunctional PEG Succinimydyl carbonate (SC-PEG); difunctional PEG propion aldehyde (A-PEG); and difunctional PEG glycidyl ether (E-PEG).
The abbreviation "dPEG" is used to encompass

35

-12-

difunctionally activated polyethylene glycols of a variety of types.

The term "chemically conjugated" as used herein means attached through a covalent chemical bond. In the practice of the invention, a synthetic hydrophilic polymer and natural polymer or derivative thereof are preferably directly bound to each other via a covalent bond(s) but may be chemically conjugated by using a linking radical, such that the synthetic polymer and natural polymer are each bound to the radical, but not directly to each other. The term "biocompatible conjugate" refers to a natural polymer chemically conjugated to a synthetic hydrophilic polymer, within the meaning of this invention. Thus, "natural polymer/PEG" (or "PEG/natural polymer") denotes a composition of the invention wherein a natural polymer is chemically conjugated to PEG. "Natural polymer/PEG" refers to a natural polymer of the invention chemically conjugated to difunctionally activated PEG, wherein the polymer molecules may be crosslinked. The synthetic polymer may be "chemically conjugated" to the natural polymer by means of a number of different types of chemical linkages. For example, the conjugation can be via an ester or a urethane linkage, but is more preferably by means of an ether linkage. An ether linkage is preferred in that it can be formed without the use of toxic chemicals and is not readily susceptible to hydrolysis in vivo.

Those of ordinary skill in the art will appreciate that synthetic polymers such as polyethylene glycol cannot be prepared practically to have exact molecular weights, and that the term "molecular weight" as used herein refers to the weight average molecular weight of a number of molecules in any given sample, as commonly used in the art. Thus, a sample of PEG 2,000 might contain a

-13-

statistical mixture of polymer molecules ranging in weight from, for example, 1,500 to 2,500 daltons, with one molecule differing slightly from the next over a range. Specification of a range of molecular weight indicates that the average molecular weight may be any value between the limits specified, and may include molecules slightly outside those limits. Thus, a molecular weight range of about 800 to about 20,000 indicates an average molecular weight of at least about 800, ranging up to about 20 kDa.

The term "available lysine residue" as used herein refers to lysine side chains exposed on the outer surface of natural polymer molecules, which are positioned in a manner to allow reaction with activated PEG. The number of available lysine residues may be determined by reaction with sodium 2,4,6-trinitrobenzenesulfonate (TNBS).

The terms "treat" and "treatment" as used herein refer to augmentation, repair, prevention, or alleviation of defects, particularly defects due to loss or absence of soft tissue or soft tissue support, or to loss or absence of bone or cartilage. Further, the term "treat" includes the use of strings of the invention to suture wounds, and the use of tubes of the invention to repair, replace, or augment a channel in the body of a living being, in particular a human being. Additionally, "treat" and "treatment" also refer to the prevention, maintenance, or alleviation of disorders or diseases using a biologically active protein coupled to and/or mixed with a conjugate-containing composition of the invention. Accordingly, treatment of soft tissue includes augmentation of soft tissue, for example, implantation of conjugates of the invention to restore normal or desirable dermal contours, as in the removal of dermal creases or furrows, or as in the replacement of

-14-

subcutaneous fat in maxillary areas where the fat is lost due to aging or in the augmentation of submucosal tissue, such as the urinary or lower esophageal sphincters. Treatment of bone and cartilage includes the use of
5 conjugates, and particularly natural polymer/PEG in combination with suitable particulate materials, to replace or repair bone tissue, for example, in the treatment of bone nonunions or fractures. Treatment of bone also includes use of conjugate-containing
10 compositions with or without additional bone growth factors. Compositions comprising conjugates with ceramic particles, preferably calcium phosphate ceramics such as hydroxyapatite and/or tricalcium phosphate, are particularly useful for the repair of stress-bearing bone
15 due to its high tensile strength.

The terms "cytokine" and "growth factor" are used to describe biologically active molecules and active peptides (which may be either naturally occurring or
20 synthetic) which aid in healing or regrowth of normal tissue. The function of cytokines and growth factors is two-fold: 1) they can incite local cells to produce new collagen or tissue, or 2) they can attract cells to the site in need of correction. As such, cytokines and growth factors serve to encourage "biological anchoring"
25 of the implant within the host tissue. As previously described, the cytokines or growth factors can either be admixed with the conjugate or chemically coupled to the conjugate. For example, one may incorporate cytokines such as interferons (IFN), tumor necrosis factors (TNF),
30 interleukins, colony stimulating factors (CSFs), or growth factors such as osteogenic factor extract (OFE), epidermal growth factor (EGF), transforming growth factor (TGF) alpha, TGF- β (including any combination of TGF- β s), TGF- β 1, TGF- β 2, platelet derived growth factor (PDGF-AA, PDGF-AB, PDGF-BB), acidic fibroblast growth factor (FGF),
35

-15-

basic FGF, connective tissue activating peptides (CTAP),
 β -thromboglobulin, insulin-like growth factors,
erythropoietin (EPO), nerve growth factor (NGF), bone
morphogenic protein (BMP), osteogenic factors, and the
5 like. Incorporation of such cytokines or growth factors,
and appropriate combinations of cytokines and growth
factors can facilitate the regrowth and remodeling of an
implant into normal tissue, or may be used in the
treatment of wounds. Further, one may chemically link
10 the cytokines or growth factors to the biocompatible
conjugate by employing a suitable amount of
multifunctional polymer molecules during synthesis. The
cytokine or growth factors may then be attached to the
free polymer ends by the same method used to attach PEG
15 to any natural polymer or derivative thereof or by any
other suitable method. By tethering cytokine or growth
factor molecules to the implant, the amount of cytokine
or growth factor required to be therapeutically effective
is substantially reduced. Conjugates incorporated with
20 cytokines or growth factors may serve as effective
controlled release drug delivery systems. By varying the
chemical linkage between the cytokine and the conjugate,
it is possible to vary the effect with respect to the
release of the cytokine or growth factor. For example,
25 when an "ester" linkage is used, the linkage is more
easily broken under physiological conditions, allowing
for sustained release of the growth factor or cytokine
from the matrix. However, when an "ether" linkage is
used, the bonds are not easily broken and the cytokine or
30 growth factor will remain in place for longer periods of
time with its active sites exposed, providing a
biological effect on the natural substrate for the active
site of the protein. It is possible to include a mixture
of conjugates with different linkages so as to obtain
35 variations in the effect with respect to the release of

-16-

the cytokine or growth factor, i.e., the sustained release effect can be modified to obtain the desired rate of release.

The term "effective amount" refers to the amount of composition required in order to obtain the effect desired. Thus, a "tissue growth-promoting amount" of a composition containing a cytokine or growth factor refers to the amount of cytokine or growth factor needed in order to stimulate tissue growth to a detectable degree. Tissue, in this context, includes connective tissue, bone, cartilage, epidermis and dermis, blood, and other tissues. The actual amount which is determined to be an effective amount will vary depending on factors such as the size, condition, sex and age of the patient and can be more readily determined by the caregiver.

The term "sufficient amount" as used herein is applied to the amount of carrier used in combination with the conjugates of the invention. A sufficient amount is that amount which, when mixed with the conjugate, renders it in the physical form desired, for example, injectable solution, injectable suspension, plastic or malleable implant, rigid stress-bearing implant, strings, tubes and so forth. Injectable formulations generally include an amount of fluid carrier sufficient to render the composition smoothly injectable, whereas malleable implants have substantially less carrier and have a clay-like consistency. Rigid stress-bearing implants may include no carrier at all and have a high degree of structural integrity. The amount of the carrier can be varied and adjusted depending on the particular conjugate used and the end result desired. Such adjustments will be apparent to those skilled in the art upon reading this disclosure.

The term "suitable particulate material" as used herein refers to a particulate material which is

-17-

substantially insoluble in water, non-immunogenic, biocompatible, and immiscible with the biocompatible conjugates of the invention. The particles of material may be fibrillar, or may range in size from about 20 to 250 μm in diameter and be bead-like or irregular in shape. Exemplary particulate materials include, without limitation, fibrillar crosslinked collagen, gelatin beads, crosslinked collagen-dPEG particles, polytetrafluoroethylene beads, silicone rubber beads, hydrogel beads, silicon carbide beads, and glass beads. Preferred particulate materials are calcium phosphates, most preferably hydroxyapatite and/or tricalcium phosphate.

The term "solid implant" refers to any solid object which is designed for insertion and use within the body, and includes bone and cartilage implants (e.g., artificial joints, retaining pins, cranial plates, and the like, of metal, plastic and/or other materials), strings which can be used as sutures or injected for soft tissue augmentation, breast implants (e.g., silicone gel envelopes, foam forms, and the like), catheters and cannulas intended for long-term use (beyond about three days), artificial organs and vessels formed from tubes of the invention (e.g., artificial hearts, pancreases, kidneys, blood vessels, and the like), drug delivery devices (including monolithic implants, pumps and controlled release devices such as Alzet® minipumps, steroid pellets for anabolic growth or contraception, and the like), sutures for dermal or internal use, periodontal membranes, lenticules, corneal shields, platinum wires for aneurysm treatment, and the like. As such, the term "solid implants" encompasses both implants formed from compositions of the biocompatible conjugates of the invention and implants made from other synthetic materials such as silicone, polyurethane, titanium,

-18-

platinum, etc., which may be coated with compositions of the invention.

The term "suitable fibrous material", as used herein, refers to a fibrous material which is

5 substantially insoluble in water, non-immunogenic, biocompatible, and immiscible with the biocompatible conjugates of the invention. The fibrous material may comprise a variety of materials having these characteristics and is combined with compositions of the

10 conjugate in order to form and/or provide structural integrity to various implants or devices such as tubes comprised of conjugates used in connection with medical and pharmaceutical uses. The conjugate compositions of the invention can be coated on the "suitable fibrous

15 material", which can then be wrapped around a bone to provide structural integrity to the bone. Thus, the "suitable fibrous material" is useful in forming the "solid implants" of the invention.

The term "in situ" as used herein means at the site

20 of administration. Thus, the injectable reaction mixture compositions are injected or otherwise applied to a site in need of augmentation and allowed to crosslink at the site of injection. Suitable sites will generally be intradermal or subcutaneous regions for augmenting dermal

25 support, at the site of bone fractures for wound healing and bone repair, and within sphincter tissue for sphincter augmentation (e.g., for restoration of continence).

The term "aqueous mixture" includes liquid

30 solutions, suspensions, dispersions, colloids, and the like containing a natural polymer and water.

The term "NFC cartilage" as used herein refers to a composition of the invention which resembles cartilage in physical consistency. NFC cartilage is prepared from

35 nonfibrillar collagen (e.g., collagen in solution) and is

-19-

crosslinked with a hydrophilic polymer, especially using dPEG. As an artifact of the production process or by design, NFC cartilage may contain about 0-20% fibrillar collagen. NFC cartilage is generally prepared by adding dPEG in acidic solution to an acidic solution of collagen, and allowing conjugation to occur prior to neutralization. The term "NFC-FC cartilage" refers to a composition similar to NFC cartilage, wherein the percentage of fibrillar collagen is about 20-80%. NFC-FC cartilage is generally prepared by adding dPEG in a neutralizing buffer to an acidic solution of collagen. The neutralizing buffer causes collagen fibril formation during the conjugation process. Similarly, "FC cartilage" refers to a composition of the invention which is prepared from fibrillar collagen and a difunctional hydrophilic polymer. FC cartilage may generally be prepared using dPEG and fibrillar collagen in neutral solutions/suspensions.

20 B. General Method

B.1 Preparation:

To form the biocompatible conjugates of the invention, a natural polymer or derivative thereof must be chemically conjugated to a synthetic hydrophilic polymer. This can be effected in a number of ways. In accordance with the preferred method, the synthetic hydrophilic polymer is activated and then reacted directly with the natural polymer. Alternatively, hydroxyl or amino groups present on the natural polymer can be activated and the activated groups will react with the polymer to form the conjugate. In accordance with a less preferred method, a linking group with activated hydroxyl or amino groups thereon can be combined with the synthetic polymer and natural polymer in a manner so as to concurrently react the natural and synthetic polymers

-20-

to form the biocompatible conjugate. Other methods of forming the biocompatible conjugates will become apparent to those skilled in the art upon reading this disclosure. Since the conjugates of the invention are to be used in the human body, it is important that all of the components, including both polymers and linking group, if used, form a conjugate that is unlikely to be reacted to or rejected by the patient. Accordingly, toxic and/or immunoreactive components are not preferred as starting materials. Some preferred starting materials and methods of forming conjugates are described further below.

Although different hydrophilic synthetic polymers can be used to form the conjugate, the polymer must be biocompatible, relatively insoluble, yet hydrophilic, and is preferably one or more forms of polyethylene glycol (PEG) due to its known biocompatibility. Various forms of PEG are extensively used in the modification of biologically active molecules because PEG can be formulated to have a wide range of solubilities and because it lacks toxicity, antigenicity, immunogenicity, and does not typically interfere with the enzymatic activities and/or conformations of peptides. Further, PEG is generally non-biodegradable and is easily excreted from most living organisms including humans.

The first step in forming the conjugates of the invention generally involves the functionalization of the PEG molecule. Various functionalized polyethylene glycols have been used effectively in fields such as protein modification (see Abuchowski et al., Enzymes as Drugs, John Wiley & Sons: New York, NY (1981) pp. 367-383; and Dreborg et al., Crit. Rev. Therap. Drug Carrier Syst. (1990) 6:315, both of which are incorporated herein by reference), peptide chemistry (see Mutter et al., The Peptides, Academic: New York, NY 2:285-332; and Zalipsky et al., Int. J. Peptide Protein Res. (1987) 30:740, both

-21-

of which are incorporated herein by reference), and the synthesis of polymeric drugs (see Zalipsky et al., Eur. Polym. J. (1983) 19:1177; and Ouchi et al., J. Macromol. Sci. -Chem. (1987) A24:1011, both of which are
5 incorporated herein by reference). Various types of conjugates formed by the binding of polyethylene glycol with specific pharmaceutically active proteins have been disclosed and found to have useful medical applications, in part due to the stability of such conjugates with
10 respect to proteolytic digestion, reduced immunogenicity, and longer half-lives within living organisms.

One form of polyethylene glycol which has been found to be particularly useful is monomethoxy-polyethylene glycol (mPEG), which can be activated by the addition of
15 a compound such as cyanuric chloride, then coupled to a protein (see Abuchowski et al., J. Biol. Chem. (1977) 252:3578, which is incorporated herein by reference). Although such methods of activating polyethylene glycol can be used in connection with certain embodiments of the
20 present invention, they are not particularly desirable in that the cyanuric chloride is relatively toxic and must be completely removed from any resulting product in order to provide a pharmaceutically acceptable composition.

Activated forms of PEG, including activated forms of
25 mPEG, can be made from reactants which can be purchased commercially. One form of activated PEG which has been found to be particularly useful in connection with the present invention is mPEG-succinate-N-hydroxysuccinimide ester (SS-PEG) (see Abuchowski et al., Cancer Biochem.
30 Biphys. (1984) 7:175, which is incorporated herein by reference). Activated forms of PEG such as SS-PEG react with proteins under relatively mild conditions and produce conjugates without destroying the specific biological activity and specificity of the protein
35 attached to the PEG. However, when such activated PEGs

-22-

are reacted with proteins, they react and form linkages by means of ester bonds. Although ester linkages can be used in connection with the present invention, they are not particularly preferred in that they undergo

5 hydrolysis when subjected to physiological conditions over extended periods of time (see Dreborg et al., Crit. Rev. Therap. Drug Carrier Syst. (1990) 6:315; and Ulbrich et al., J. Makromol. Chem. (1986) 187:1131, both of which are incorporated herein by reference).

10 It is possible to link PEG to proteins via urethane linkages, thereby providing a more stable attachment which is more resistant to hydrolytic digestion than the ester linkages (see Zalipsky et al., Polymeric Drug and Drug Delivery Systems, Chapter 10, "Succinimidyl
15 Carbonates of Polyethylene Glycol" (1991) incorporated herein by reference to disclose the chemistry involved in linking various forms of PEG to specific biologically active proteins). The stability of urethane linkages has been demonstrated under physiological conditions (see
20 Veronese et al., Appl. Biochem. Biotechnol. (1985) 11:141; and Larwood et al., J. Labelled Compounds Radiopharm. (1984) 21:603, both of which are incorporated herein by reference). Another means of attaching the PEG to a protein can be by means of a carbamate linkage (see
25 Beauchamp et al., Anal. Biochem. (1983) 131:25; and Berger et al., Blood (1988) 71:1641, both of which are incorporated herein by reference). The carbamate linkage is created by the use of carbonyldiimidazole-activated PEG. Although such linkages have advantages, the
30 reactions are relatively slow and may take 2 to 3 days to complete.

The various means of activating PEG described above and publications (all of which are incorporated herein by reference) cited in connection with the activation means
35 are described in connection with linking the PEG to

-23-

specific biologically active proteins, and not to inert, biologically inactive, natural polymers. (See Polymeric Drug and Drug Delivery Systems, Chapter 10, "Succinimidyl Carbonates of Polyethylene Glycol" (1991), incorporated
5 herein by reference to disclose the chemistry involved in linking various forms of PEG to specific biologically active proteins.) Collagen has also been bound to PEG via an ester linkage as taught by U.S. Patent 5,162,430 issued Nov. 10, 1992, also incorporated herein by
10 reference. The present invention now discloses that activated PEG compounds can be used in connection with the formation of a wide range of inert, biocompatible conjugates held together by a variety of different types of bonds. Such conjugates provide a range of improved,
15 unexpected characteristics and as such can be used to form the various compositions and articles of the present invention.

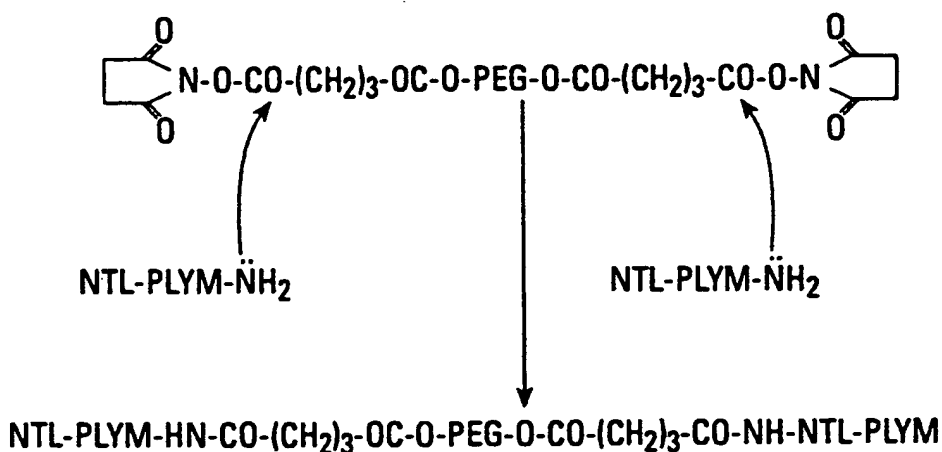
B.2 Specific Forms of Activated PEG.

20 As indicated above, the conjugates of the present invention can be prepared by covalently binding a variety of different types of synthetic hydrophilic polymers to a natural polymer or derivatives thereof. However, in that the final product or conjugate obtained must have a
25 number of characteristics, such as being biocompatible and non-immunogenic, it has been found that it is useful to use polyethylene glycol as the synthetic hydrophilic polymer. The polyethylene glycol must be modified in order to provide activated groups on one or preferably
30 both ends of the molecule so that covalent binding can occur between the PEG and the natural polymer. Some specific functionalized forms of PEG are shown structurally below, as are the reaction products obtained by reacting these functionalized forms of PEG with a
35 natural polymer or derivative thereof.

-24-

1. PEG Conjugation of Natural Polymers:

The first functionalized PEG is difunctionalized PEG succinimidyl glutarate, referred to herein as (SG-PEG). The structural formula of this molecule and the reaction product obtained by reacting it with a natural polymer (represented by NTL-PLYM) with an amine group NH_2 , thereon is shown below in Formula 1:

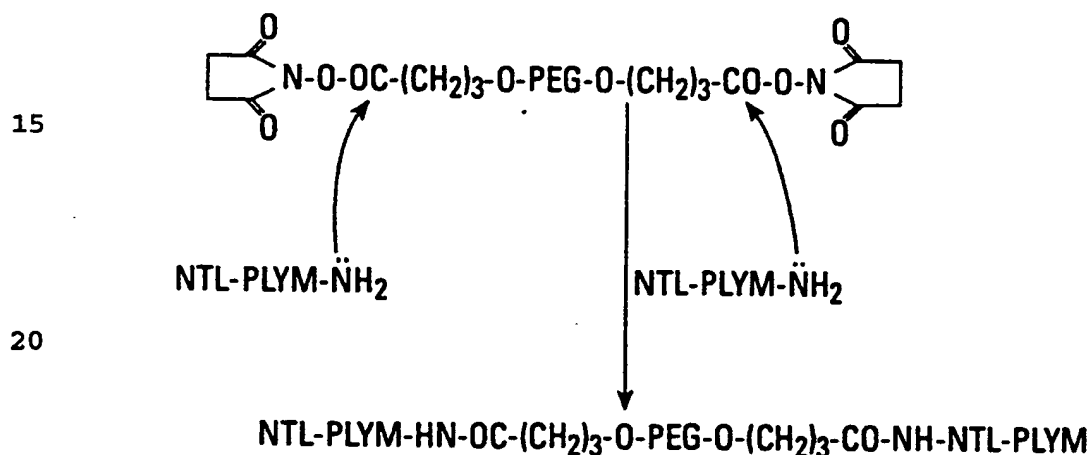
SG-PEG: Difunctional PEG Succinimidyl Glutarate**FORMULA 1**

Another difunctionally activated form of PEG is referred to as PEG succinimidyl (S-PEG). The structural formula for this compound and the reaction product obtained by reacting it with a natural polymer is shown below. It should be noted that the methyl group is repeated three times on each side of the molecule. In a general structural formula for this compound, the subscript 3 is replace with an "n". In the specific embodiments of Formulas 1 and 2, $n=3$, in that there are

-25-

three repeating CH_2 groups on each side of the PEG. The structure of Formula 2 includes an "ether" linkage between the difunctionalized PEG and the natural polymers on each end which ether linkage is not subject to hydrolysis. This is distinct from the conjugate of Formula 1, wherein an ester linkage is provided. The ester linkage is subject to hydrolysis under physiological conditions.

10 S-PEG, n=3: Difunctional PEG Succinimidyl



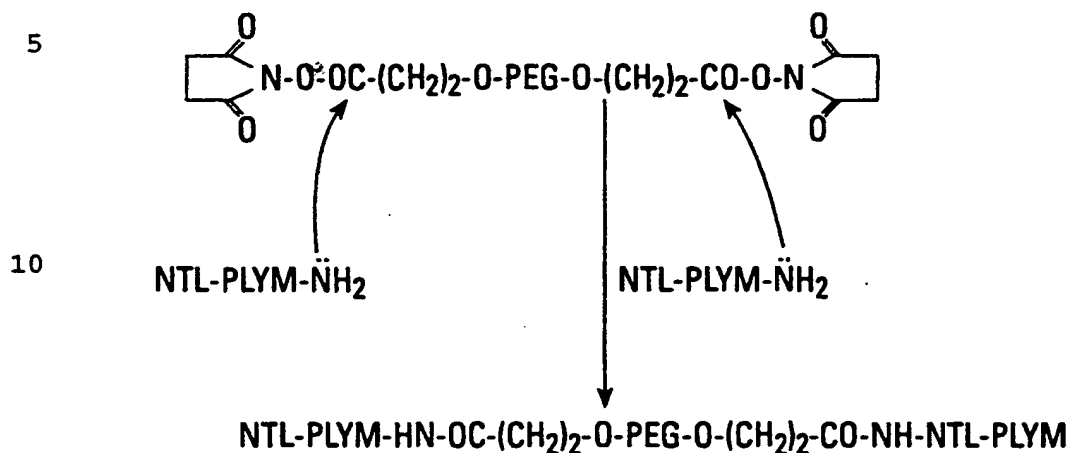
25

FORMULA 2

Yet another derivatized form of polyethylene glycol, wherein n=2, is shown in Formula 3, as is the conjugate formed by reacting the derivatized PEG with collagen.

30

35

S-PEG, n=2: Difunctional PEG Succinimidyl

FORMULA 3

20 Another preferred embodiment of the invention similar to the conjugates provided in Formulas 2 and 3 is provided when n=1. The structural formula and resulting conjugate are shown in Figure 4. It is noted that the conjugate includes both an ether and a peptide linkage. These

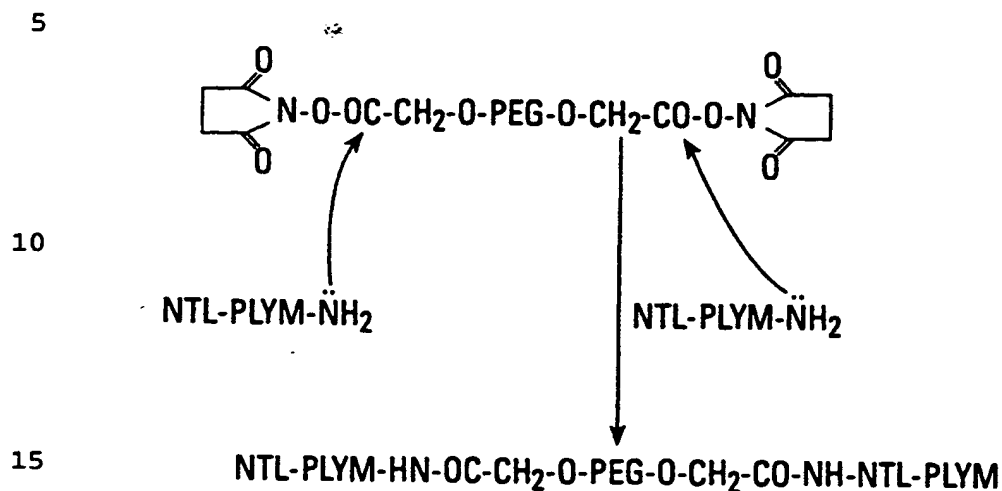
25 linkages are stable under physiological conditions.

30

35

- 27 -

S-PEG, n=1: Difunctional PEG Succinimidyl



FORMULA 4

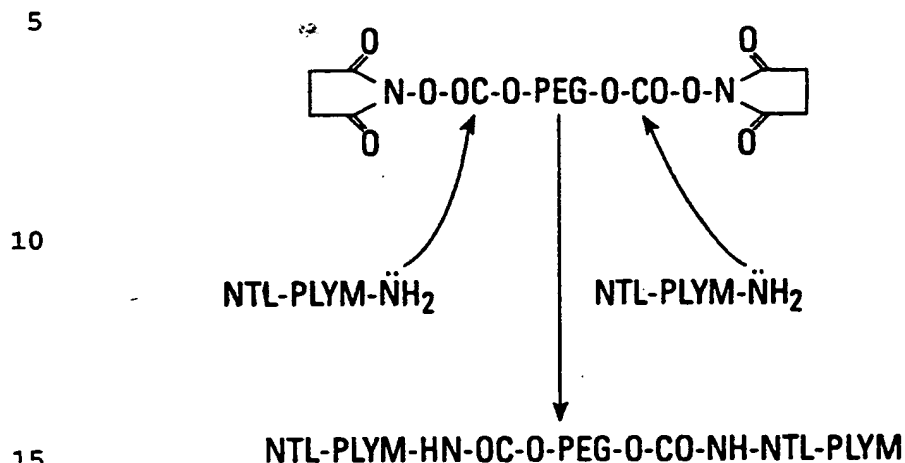
20 Yet another derivatized form of PEG is provided when n=0. The difunctionalized form is referred to as PEG succinimidyl carbonate (SC-PEG). The structural formula of this compound and the conjugate formed by reacting

25 this conjugate includes a urethane linkage, the conjugate has been found not to have a high degree of stability under physiological conditions.

30

35

-28-

SC-PEG, n=0: Difunctional PEG Succinimidyl Carbonate**FORMULA 5**

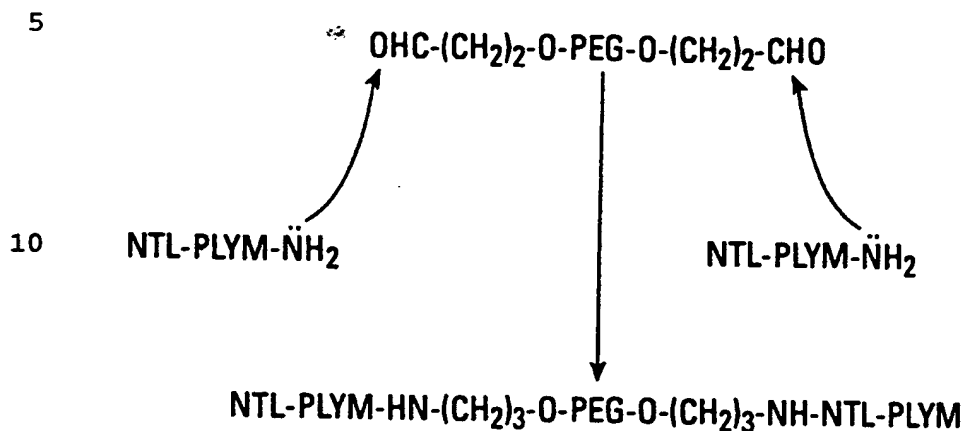
20 All of the above derivatives involve the inclusion of the succinimidyl group. However, different activating groups can be attached to one or both ends of the PEG. For example, the PEG can be derivatized to form difunctional PEG propion aldehyde (A-PEG), which is shown

25 in Formula 6, as is the conjugate formed by the reaction of A-PEG with a natural polymer or derivative thereof.

30

35

- 29 -

A-PEG: Difunctional PEG Propion Aldehyde**FORMULA 6**

20 Yet another functionalized form of polyethylene glycol is difunctional PEG glycidyl ether (E-PEG), which is shown in Formula 7, as is the conjugate formed by reacting such with a natural polymer or derivative thereof.

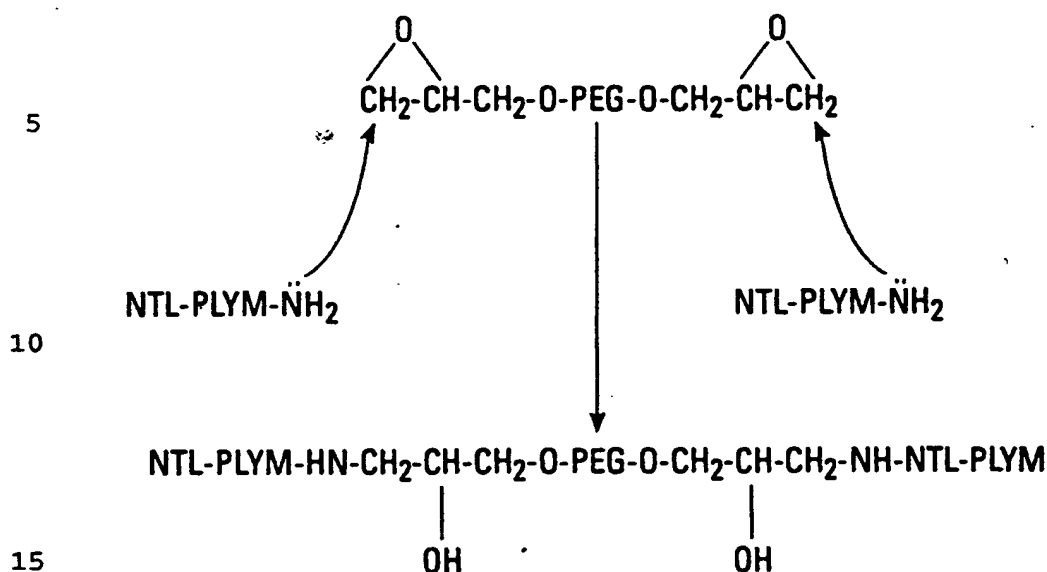
25

30

35

- 30 -

E-PEG: Difunctional PEG Glycidyl Ether



FORMULA 7

20 The conjugates formed using the functionalized forms of PEG vary depending on the functionalized form of PEG used in the reaction. Further, the final product can be varied with respect to its characteristics by changing the molecular weight of the PEG. In general, the

25 stability of the conjugate is improved by eliminating any ester linkages between the PEG and the natural polymer and including ether and/or urethane linkages. In certain situations, it is desirable to include the weaker ester

30 linkages so that the linkages are gradually broken by hydrolysis under physiological conditions, breaking apart the matrix and releasing a component held therein, such as a growth factor or cytokine. By varying the chemical structure of the linkages, the rate of sustained release can be varied.

35

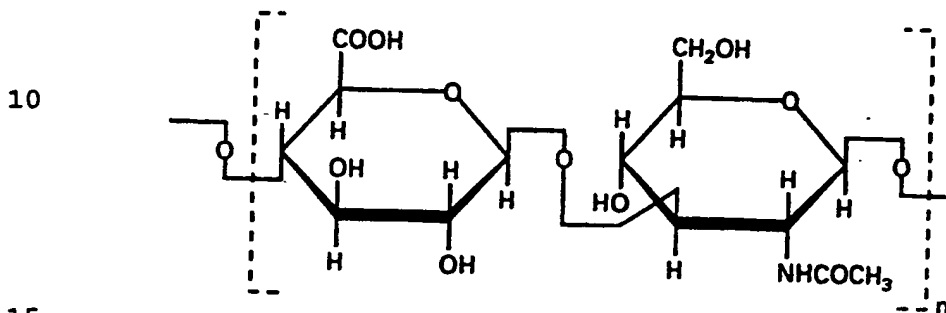
-31-

2. PEG Conjugation of Polysaccharides:

Hyaluronic acid comprises a polymer of the following repeating monomeric units, as shown in Formula 8.

5

HYALURONIC ACID



Alternating units of 1,4-linked
N-acetylglucosamine and glucuronic acid

FORMULA 8

20

Hyaluronic acid can be conjugated to PEG using a number of different methods. Preferred methods include modification of the carboxyl group and modification of the acetyl group. A reaction scheme showing a modification of a carboxyl group of hyaluronic acid using PEG-hydrazine is shown in figure 8. A reaction scheme showing a modification of an acetyl group with succinimydyl-PEG (S-PEG) is shown in figure 9.

30

3. PEG Conjugation of Proteoglycans:

As shown in figure 10, chondroitin sulfate A, chondroitin sulfate C, and dermatan sulfate (chondroitin sulfate B) are of similar structure to hyaluronic acid and can be conjugated to PEG by creating a derivative

35

using much the same methods used to derivatize hyaluronic acid.

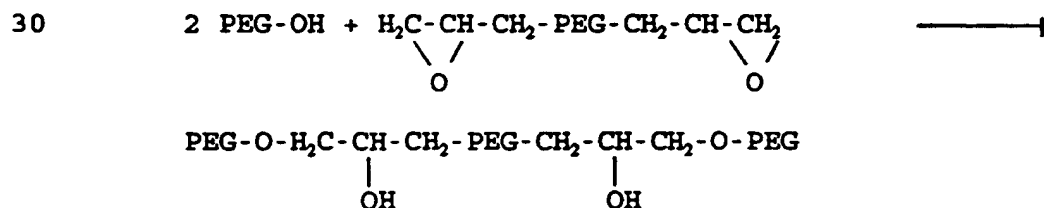
4. PEG Conjugation of Polylactic Acid:

5 Esters undergo nucleophilic substitution, which is
typical of carboxylic acid derivatives. Attack occurs at
the electron-deficient carbonyl carbon. These reactions
are sometimes carried out in the presence of acid. In
these acid-catalyzed reactions, H^+ attaches itself to the
10 oxygen of the carbonyl group, rendering the carbonyl
carbon even more susceptible to nucleophilic attack. A
reaction scheme demonstrating such is shown in figure 11.

5. PEG Conjugation of Polyethylene:

15 A number of different activated PEGs, both
difunctional and multifunctional, can be used to
crosslink polyethylene. Difunctional or multifunctional
E-PEG can be used for crosslinking polyethylene due to
the ease of opening the highly strained three-member
20 ring. The bond angles of the ring, which average 60
degrees, are considerably smaller than the normal
tetrahedral carbon angles of 109 degrees. Because
polyethylene is not readily denatured at elevated
temperatures (as are many natural polymers, such as
25 collagen), E-PEG may be used to crosslink polyethylene at
elevated temperatures.

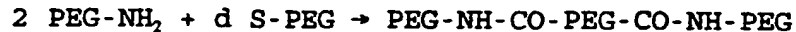
The reaction of difunctional E-PEG with polyethylene is shown below:



35

-33-

The reaction of difunctional S-PEG with polyethylene is shown below:



5

The reactions of polyethylene with a multifunctional activated form of E-PEG and S-PEG are shown, respectively, within figures 12 and 13.

10 The crosslinking reaction between the natural polymer and synthetic polymer may be performed in vitro, or a reaction mixture may be injected for crosslinking in situ. At sufficient density, crosslinked biocompatible conjugates resemble cartilage, and are useful as substitutes therefor (e.g., cranial onlay, ear and nose reconstruction, and the like). In addition to forming
15 conjugates between natural polymers, multifunctional polymers may also be used to covalently bind natural polymers to other proteins, particularly cytokines or growth factors, for compositions particularly suited for wound healing, osteogenesis, and immune modulation. Such
20 tethering of growth factors or cytokines to biocompatible polymers such as collagen provides an effective slow-release drug delivery system. Ether linkages might be used to bond the natural and synthetic polymers whereas
25 ester linkages might be used to attach a cytokine or growth factor and thereby obtain slow release of the cytokine or growth factor.

6. PEG Conjugation of Collagen:

30 Suitable collagens for use in the present invention include all types of collagen, including natural telopeptide-containing collagen which may be used in situations where immune response sensitivity is not significant. However, for most applications, non-
35 immunogenic atelopeptide collagen, in particular types I,

-34-

II and III, is preferred. Collagens may be soluble (for example, commercially available Vitrogen® 100 collagen-in-solution) and may or may not have the telopeptide regions. Preferably, the collagen will be reconstituted

5 fibrillar atelopeptide collagen, for example, Zyderm® Collagen Implant (ZCI) or atelopeptide collagen in solution (CIS). Various forms of collagen are available commercially, or may be prepared by the processes described in, for example, U.S. Pat. Nos. 3,949,073;

10 4,488,911; 4,424,208; 4,582,640; 4,642,117; 4,557,764; and 4,689,399, all incorporated herein by reference. Non-fibrillar, atelopeptide, reconstituted collagen is preferred in order to form certain products. Methods for conjugating collagen to synthetic hydrophilic

15 polymers such as PEG are described in detail in U.S. Patent No. 5,162,430.

The compositions of the invention comprise natural polymers or derivatives thereof chemically conjugated to a selected synthetic hydrophilic polymer or polymers.

20 Natural polymers such as collagen derivatives contain a number of available amino and hydroxy groups which may be used to bind the synthetic hydrophilic polymer. The polymer may be bound using a "linking group", as the native hydroxy or amino groups on the natural polymer and

25 synthetic polymer frequently require activation before they can be linked. For example, one may employ compounds such as dicarboxylic anhydrides (e.g., glutaric or succinic anhydride) to form a polymer derivative (e.g., succinate), which may then be activated by

30 esterification with a convenient leaving group, for example, N-hydroxysuccinimide, N,N'-disuccinimidyl oxalate, N,N'-disuccinimidyl carbonate, and the like. See also Davis, U.S. Pat. No. 4,179,337, for additional linking groups. Presently preferred dicarboxylic

35 anhydrides that are used to form polymer-glutarate

-35-

compositions include glutaric anhydride, adipic anhydride, 1,8-naphthalene dicarboxylic anhydride, and 1,4,5,8-naphthalenetetracarboxylic dianhydride. The polymer thus activated is then allowed to react with the natural polymer to form a biocompatible conjugate of the invention.

Conjugates with Ester Linkages

In one embodiment, a pharmaceutically pure form of monomethylpolyethylene glycol (mPEG) (mw 5,000) is reacted with glutaric anhydride (pure form) to create mPEG glutarate. The glutarate derivative is then reacted with N-hydroxysuccinimide to form a succinimidyl monomethylpolyethylene glycol glutarate. The succinimidyl ester (mPEG*, denoting the activated PEG intermediate) is then capable of reacting with free amino groups (lysine residues) present on certain natural polymers. The reaction results in a natural polymer-PEG conjugate of the invention wherein one end of the PEG molecule is free or nonbound. Other polymers may be substituted for the monomethyl PEG, as described above. Similarly, the coupling reaction may be carried out using any known method for derivatizing proteins and synthetic polymers. The number of available lysines conjugated may vary from a single residue to 100% of the lysines, preferably 10%-50%, and more preferably 20-30%. The number of reactive lysine residues may be determined by standard methods, for example by reaction with TNBS.

By using a difunctionalized PEG, it is possible to connect the same or a different natural polymer or cytokine to the other end of the PEG. Further, the bonds connecting the PEG to any natural polymer and/or other molecules may be ester, ether, and/or urethane bonds and are preferably ether bonds.

-36-

Compositions for Bone Repair

Formulations suitable for repair of bone defects or nonunions may be prepared by providing high concentration compositions of biocompatible conjugates, optionally in
5 admixture with suitable particulate materials. When making bone repair compositions, the linkage between the collagen and polymer is preferably an ether linkage in order to avoid deterioration due to the hydrolysis of the ester linkages. Such conjugate/particulate compositions
10 may be malleable or rigid, depending on the amount of liquid incorporated. Formulations for treatment of stress-bearing bone are preferably dried and rigid, and will generally comprise between about 45% and 85% particulate calcium phosphate mineral, for example
15 hydroxyapatite or tricalcium phosphate, or mixtures thereof. The tensile strength and rigidity may be further increased by heating the composition under vacuum at about 60-90°C, preferably about 75°C, for about 5 to 15 hours, preferably about 10 hours. Malleable
20 compositions may be used for repair of non-stressed bone or cartilage.

The activated mPEG* may be replaced, in whole or in part, by difunctionally activated PEG (dPEG*, e.g., non-methylated PEG which is then activated at each end), thus
25 providing a crosslinked or partially crosslinked composition. Such compositions are, however, quite distinct from conventionally crosslinked collagen compositions (e.g., using heat, radiation, glutaraldehyde, and the like), as the long-chain
30 synthetic hydrophilic polymer imparts a substantial hydrophilic character to the composition. In a presently preferred embodiment, approximately 1-20% of the PEG is difunctionally activated PEG. The character of the composition may be adjusted as desired, by varying the
35 amount of difunctionally activated PEG included.

-37-

In another presently preferred embodiment, difunctionally activated PEG* (substantially 100% at pH 7) is used to crosslink a natural polymer or derivative thereof. In one version, CIS (about 3-100 mg/mL, preferably about 10-40 mg/mL) is allowed to react with dPEG* (difunctional PEG activated at each end by addition of an acid anhydride having a leaving group such as succinimide) having a molecular weight of about 2,000 to about 100,000 (preferably about 3,400-20,000), which is added as a concentrated solution to a final reaction mixture concentration of about 5-40%, preferably about 10-20%. This represents a 5- to 10-fold excess of dPEG* to collagen on a molar basis. The collagen molecules bind to dPEG* without mechanical mixing or agitation and settle out of solution to produce a cartilaginous collagen-polymer conjugate containing approximately 20-80% fibrillar collagen. The conjugate is then washed with PBS to remove any remaining unreacted dPEG* to provide the material of the invention. A cartilaginous collagen-polymer conjugate may also be prepared by mixing a dPEG* solution (pH 3) with collagen-in-solution between two syringes to homogeneity, then casting into a suitable container (e.g., a Petri dish). A 20% w/v dPEG* solution (pH 7) is then added to the non-fibrillar collagen-PEG solution to result in a lightly cartilaginous fibrillar collagen-polymer conjugate. The resulting NFC-FC conjugate cartilage contains approximately 1-40% fibrillar collagen.

The characteristics of the final product may be adjusted by varying the initial reactants and reaction conditions. For example, natural polymers other than collagen can be used. In general, increased concentrations of the natural polymer or the PEG provide a denser, less porous product. By varying the pH of the collagen solution and the dPEG* solution, compositions

-38-

may be producing over a wide range of fibrillar collagen content. If desired, the denser formulations may be cast or molded into any shape desired, for example into sheets, membranes, tubes, cylinders, strings, cords, ropes, and the like. Certain shapes may be produced by extrusion.

Breast Implants

Biocompatible polymer conjugates can also be used as coatings for breast implants. The surface of a standard silicone-shell implant can be chemically derivatized to provide active binding sites for di- or multifunctional PEG bound to a natural polymer resulting in a three-part conjugate as follows: (natural polymer-PEG-silicone). The presence of the conjugate coating bound directly to the silicone via PEG will serve to reduce scar tissue formation and capsular contracture. Unlike typical coated breast implants, scar tissue will not be able to grow between the conjugate coating and the surface of the implant itself.

Alternatively, the conjugate can be formed into a hollow sphere for use as a breast implant shell. The shell can then be filled with a radiolucent material, such as triglycerides, to facilitate mammography.

Coated Medical Devices

The injectable conjugate formulations (gels or solutions) may be used to coat implants, catheters, tubes (e.g., for blood vessel replacement), meshes (e.g., for tissue reinforcement), strings, and the like. Biocompatible conjugate formulations can also be used to coat platinum wires, which can then be administered to the site of an aneurysm via catheter. Gels may be prepared with various polymer concentrations and different reaction times. CIS is the preferred starting

-39-

material when the desired properties are high density, rigidity, viscosity, and translucence. However, one may substitute fibrillar collagen (preferably atelopeptide fibrillar collagen such as ZCI), as well as other natural
5 polymers, to obtain products having different characteristics, such as greater opacity, flexibility, and susceptibility to colonization by cells following implantation. CIS-based materials are presently preferred for coating articles designed for implantation,
10 such as catheters and stress-bearing bone implants. The CIS material is linked to the PEG by an ether bond.

Compositions of the invention (particularly crosslinked collagen compositions) are also useful for coating articles for implantation or relatively long-term
15 residence within the body. Such surface treatment renders the object non-immunogenic and, as such, reduces the incidence of foreign body reactions. Accordingly, one can apply compositions of the invention to catheters, cannulas, bone prostheses, cartilage replacements, breast
20 implants, minipumps, and other drug delivery devices, artificial organs, and the like. Application may be accomplished by dipping the object into the reaction mixture while crosslinking is occurring and allowing the adherent viscous coating to dry. One may pour, brush, or
25 otherwise apply the reaction mixture if dipping is not feasible. Alternatively, one may use flexible sheets or membranous forms of collagen-polymer conjugate to wrap the object with, sealing corners and edges with the reaction mixture.

30 In another embodiment, the object may be dipped in a viscous collagen-in-solution bath, or in a fibrillar collagen solution until the object is completely coated. The collagen solution is fixed to the object by dipping the collagen-coated object into a dPEG* (pH 7) solution
35 bath, then allowing the collagen-polymer coated object to

-40-

dry. Alternatively, viscous collagen-in-solution is mixed with a dPEG* (pH 3) solution and polymerized rapidly, as described above. The object is dipped in the acidic collagen-polymer solution and cured by dipping the coated object into a neutralizing buffer containing about 20% by weight dPEG* (pH 7), resulting in a collagen-polymer coated object.

Coated Implants

In addition to breast implants, the biocompatible conjugates of the present invention can be used to produce a variety of different types of coated implants. The conjugates can be formed such as by binding a synthetic polymer such as PEG to a natural polymer such as hyaluronic acid. The biocompatible conjugate formed can be coated onto the surface of any type of implant device and will be useful in improving the biocompatibility of the implant. It is also possible to use multifunctional PEG. When multifunctional PEG is used, another active site on the PEG can be used to bind a biologically active compound such as a cytokine. When the three-part conjugate is formed, it can be coated onto the surface of the implant. The inclusion of a conjugate comprised of cytokine-PEG-hyaluronic acid on the surface of the implant promotes integration of growth of the surrounding cells into the implant.

In accordance with a preferred embodiment of the coated implant, a multifunctional synthetic polymer such as multifunctional PEG is used. One of the active sites of the multifunctional PEG is connected to a natural polymer such as hyaluronic acid or collagen while another active site of the multifunctional PEG is allowed to react directly with an activated site on the surface of the implant. Accordingly, the conjugate is covalently bound directly to the surface of the implant. It is also

-41-

pointed out that many implants are used to augment or repair bone and require a tight fit within the space where the implant is to be placed. When such is the case, it is desirable to create a coated implant wherein the conjugate used for the coating is initially comprised of a large amount of water, e.g., a collagen-PEG conjugate. When a collagen-PEG conjugate is produced and coated on the surface of the implant, the coating can then be dried, which will reduce the size of the coating substantially. The coated implant is then placed in position within a bone to be repaired or augmented and will rehydrate and expand in tissue. In addition to combining the coating with various biologically active compounds such as cytokines and growth factors, it is possible to include particular materials which can aid in improving the structural integrity of the coating and provide for a more irregular surface to assist in promoting integration with the surrounding tissues. Coated implants are preferably coated with a conjugate formed with ether linkages in that it is desirable to maintain the conjugate when used in a living body, and the ether linkages are less susceptible to hydrolysis than the ester linkage.

25 Conjugate and Cytokines and/or Growth Factors

Compositions of the invention containing biologically active cytokines or growth factors such as EGF and TGF- β are prepared by mixing an appropriate amount of the cytokine or growth factor into the composition, or by combining the cytokine or growth factor with a natural polymer prior to treatment with activated PEG. By employing an appropriate amount of difunctionally activated PEG, a degree of crosslinking may be established, along with conjugates consisting of a

35

-42-

natural polymer bound to a cytokine or growth factor by a synthetic hydrophilic polymer.

Preferably, the cytokine or growth factor is first reacted with a molar excess of dPEG* in a dilute solution over a 3 to 4 hour period. The cytokine or growth factor is preferably provided at a concentration of about 1 $\mu\text{g/mL}$ to about 5 mg/mL , while the dPEG* is preferably added to a final concentration to provide a 30- to 50-fold molar excess. The resulting conjugated cytokine is then added to an aqueous collagen mixture (about 1 to about 60 mg/mL) at pH 7-8 and allowed to react further. The resulting composition is allowed to stand overnight at ambient temperature. The pellet is collected by centrifugation and is washed with PBS by vigorous vortexing in order to remove any non-bound cytokine or growth factor molecules.

Biocompatible Strings

In one embodiment of the invention, the biocompatible conjugates are used to form elongated cylinders or strings. The strings have a diameter and range of about 0.10mm to about 20mm and more preferably a diameter of about 0.25mm to about 2.5mm. The strings may be any length, but preferably have a length in the range of about 0.25cm to about 25cm. The length and diameter of the string will depend, to a large extent, upon the desired use. The strings may be produced by molding or extrusion of the conjugate material. Strings which dissolve in tissue could be used as surgical sutures and would be comprised of conjugates using ester linkages which are broken by hydrolysis. The strings can be cut into small pieces, dehydrated and injected for soft tissue augmentation. The strings can be modified to include other biologically active components such as

35

-43-

cytokines or growth factors to encourage further tissue deposition for soft tissue augmentation.

Membranous Forms

5 Flexible sheets or membranous forms may be prepared by methods known in the art, for example, U.S. Patent Nos. 4,600,533; 4,412,947; and 4,242,291. These methods can be used to produce membranes using the biocompatible conjugates of the present invention. To
10 produce a membrane, a natural polymer such as a high concentration (10-100 mg/mL) CIS or fibrillar collagen (preferably atelopeptide fibrillar collagen such as ZCI) is cast into a flat sheet container. A solution of mPEG* (having a molecular weight of approximately 5,000) is
15 added to the cast collagen solution and allowed to react overnight at room temperature. The resulting collagen-polymer conjugate is removed from the reaction solution using a sterile spatula or the like and washed with PBS to remove excess unreacted mPEG*.

20 The resulting conjugate may then be compressed under constant pressure to form a uniform flat sheet or mat, which is then dried to form a membranous implant of the invention. More flexible membranous forms are achieved by using lower concentrations of the natural polymer
25 (e.g., collagen) and high synthetic polymer concentrations.

 Less flexible membranous forms are prepared by using a dPEG* solution rather than mPEG*. CIS, at room temperature, is mixed with a buffer solution and
30 incubated at 37°C overnight. The resulting gel is compressed under constant pressure, dried, and desalted by washing. The resulting membrane is then crosslinked by treating with dPEG*, washed, and then dried at low temperature.

35

-44-

Alternatively, CIS or fibrillar collagen (10-100 mg/mL) is cast into a flat sheet container. A solution of dPEG* (22-50% w/v) is added to the cast collagen. The mixture is allowed to react over several hours at room temperature. Shorter reaction times result in more flexible membranes. The resulting collagen-polymer membrane may be optionally dehydrated under a vacuum oven or by lyophilization or air-drying.

10 Sponges

Biocompatible conjugates may also be prepared in the form of sponges by lyophilizing an aqueous slurry of the composition following conjugation.

15 Biocompatible Polymer Tubes

The biocompatible conjugates of the present invention can be used to form tubes by molding or extrusion. The tubes have an outer diameter in the range of 0.25mm to about 5.0cm and inner diameter in the range of 0.05mm to about 4.9cm. The tubes have a generally circular cross section with respect to their inner and outer diameters. Although the tubes may be of any length, they generally have a length of more than 10mm and, more preferably, greater than 10cm. Although the tubes may be produced with the conjugates having any type of linkages, including ester, ether, or urethane linkages, it is preferable to produce the tubes using ether linkages. The tubes can be used to repair various types of channel in a living being such as veins, arteries, and fallopian tubes. However, the use of the tubes is not limited as such.

Soft Tissue Augmentation

Compositions of the invention have a variety of uses. Malleable, plastic compositions may be prepared as

-45-

injectable formulations which are suitable for dermal augmentation, for example, for filling in dermal creases and providing support for skin surfaces. Such compositions are also useful for augmenting sphincter tissue, (e.g., for restoration of continence). In such cases, the formulation may be injected directly into the sphincter tissue to increase bulk and permit the occluding tissues to meet more easily and efficiently. These compositions may be homogeneous or may be prepared as suspensions of small microgel conjugate particles or beads which are delivered in a nonaqueous fluid carrier. The beads/particles rehydrate and swell in situ. This has the advantage over commercial preparations in that less volume of product needs to be injected to achieve the desired connection.

Surprisingly, one may administer the reaction mixture by injection before crosslinking has completed. For example, an aqueous collagen mixture is combined with a low-concentration dPEG* solution, mixed, and the combination injected or applied before the viscosity increases sufficiently to render injection difficult (usually about 20 minutes). Mixing may be accomplished by passing the mixture between two syringes equipped with Luer lock hubs, or through a single syringe having dual compartments (e.g., double barrel). The composition crosslinks in situ, and may additionally crosslink to the endogenous tissue, anchoring the implant in place. In this method, one can use collagen (preferably fibrillar collagen) at a concentration of about 10-100 mg/mL, although about 30-80 mg/mL is preferred, most preferably about 33 mg/mL. The dPEG* concentration is preferably about 0.1 to about 3%, although concentrations as high as 30% may be used if desired. The mixture is injected directly into the site in need of augmentation, and causes essentially no inflammation or foreign body

-46-

reaction. One may additionally include particulate materials in the collagen reaction mixture, for example, hydrogel or collagen-dPEG beads, or hydroxyapatite/tricalcium phosphate particles, to provide a bulkier or
5 more rigid implant after crosslinking.

Cartilage Repair

Compositions of the invention may be prepared in a form that is dense and rigid enough to substitute for
10 cartilage. These compositions are useful for repairing and supporting tissue which require some degree of structure, for example, in reconstruction of the nose, ear, knee, larynx, tracheal rings, and joint surfaces. One can also replace tendons, ligaments, and blood
15 vessels using appropriately formed cartilaginous material. In these applications, the material is generally cast or molded into shape. In the case of tendons and ligaments, it may be preferable to form filaments for weaving into cords or ropes. A reinforcing
20 mesh (e.g., nylon or the like) may optionally be incorporated to increase structural integrity.

Cytokine and Growth Factor Administration

Compositions of the invention containing cytokines
25 and growth factors are particularly suited for sustained administration, as in the case of wound healing promotion. Osteoinductive factors and cofactors (including TGF- β) and bone morphogenic protein (BMP) may advantageously be incorporated into compositions destined
30 for bone replacement, augmentation, and/or defect repair. Compositions provided in the form of a membrane may be used to wrap or coat transplanted organs in order to suppress rejection and induce improved tissue growth. Similarly, organs may be coated for transplantation using
35 a crosslinking reaction mixture of growth factor-polymer

-47-

conjugates and collagen. Alternatively, one may administer antiviral and antitumor factors such as TNF, interferons, CSFs, TGF- β , and the like for their pharmaceutical activities. The amount of composition
5 used will depend upon the severity of the condition being treated, the amount of factor incorporated in the composition, the rate of delivery desired, and the like. However, these parameters may easily be determined by routine experimentation, for example, by preparing a
10 model composition following the examples below, and assaying the release rate of active compound in a suitable experimental model.

EXAMPLES

15 The following examples are set forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make the conjugates, formulations, articles and implants incorporating such conjugates and are not intended to
20 limit the scope of the invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, molecular weight, etc.), but some experimental errors and deviation should be accounted for. Unless indicated otherwise, parts are parts by
25 weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1

30 (Preparation of Collagen-PEG)
(A) Monomethyl-PEG 5000 (50 g, 10 mmol, Aldrich Chemical Co.) is dissolved in 1,2-dichloroethane (250 mL) and heated at reflux with glutaric anhydride (5 g) and pyridine (4 mL) under nitrogen for 3 days. The solution
35 is then filtered and the solvent evaporated, and the

-48-

residue dissolved in water (100 mL) and washed with diethyl ether (2X 50 mL). The resulting PEG-glutarate is extracted from the water with chloroform (2X 50 mL), and the chloroform evaporated to yield about 43 g of PEG-glutarate. The PEG-glutarate is then dissolved in dimethylformamide (DMF, 200 mL) at 37°C, and N-hydroxysuccinimide (10% molar xs) added. The solution is cooled to 0°C, and an equivalent amount of dicyclohexylcarbodiimide added in DMF solution (10 mL). The mixture is left at room temperature for 24 hours, and then filtered. Cold benzene (100 mL) is then added, and the PEG-succinimidyl glutarate (PEG-SG) precipitated by adding petroleum ether (200 mL) at 0°C. The precipitate is collected on a sintered glass filter. Dissolution in benzene, followed by precipitation with petroleum ether is repeated three times to provide "activated" PEG (PEG-SG).

Vitrogen 100® collagen in solution (400 mL, 1.2 g collagen, 0.004 mmol) was mixed with 0.2 M phosphate buffer (44 mL) to elevate the pH to 7.4. Next, a three-fold molar excess of SG-PEG (6.00 g, 1.2 mmol) was dissolved in water for injection (40 mL) and sterile-filtered. The SG-PEG solution was then added to the collagen solution, and the mixture allowed to stand at 17-22°C for about 15 hours. The solution was then centrifuged, and the resulting pellet (25 g) of reconstituted fibrils collected and washed with phosphate-buffered saline (PBS, 3X 400 mL) to remove residual PEG. The resulting material has a solid, coherent elasticity, and may be picked up on a spatula (the equivalent non-conjugated collagen, Zyderm® Collagen Implant, is more fluid). The resulting material may be diluted with PBS to provide a dispersion having a concentration of 20.5 mg/mL collagen-PEG.

35

-49-

(B) Similarly, proceeding as in part (A) above but substituting polypropylene glycol and POE-POP block polymers for polyethylene glycol, the corresponding collagen-PPG and collagen-POE-POP compositions are prepared.

(C) Difunctional PEG 3400 (34 g, 10 mmol, Aldrich Chemical Co.) is dissolved in 1,2-dichloroethane (250 mL) and heated at reflux with glutaric anhydride (10 g) and pyridine (4 mL) under nitrogen for 3 days. The solution is then filtered and the solvent evaporated, and the residue dissolved in water (100 mL) and washed with diethyl ether (2X 50 mL). The resulting PEG-diglutarate is extracted from the water with chloroform (2X 50 mL), and the chloroform evaporated to yield PEG-diglutarate. The PEG-diglutarate is then dissolved in DMF (200 mL) at 37°C, and N-hydroxysuccinimide (10% molar xs) added. The solution is cooled to 0°C, and an equivalent amount of dicyclohexylcarbodiimide added in DMF solution (10 mL). The mixture is left at room temperature for 24 hours, and then filtered. Cold benzene (100 mL) is then added, and the PEG-di(succinimidyl glutarate) (dPEG-SG) precipitated by adding petroleum ether (200 mL) at 0°C. The precipitate is collected on a sintered glass filter. Dissolution in benzene, followed by precipitation with petroleum ether is repeated three times to provide "activated" dPEG (dPEG*).

Vitrogen 100® collagen in solution (400 mL, 1.2 g collagen, 0.004 mmol) was mixed with 0.2 M phosphate buffer (44 mL) to elevate the pH to 7.4. Next, a three-fold molar excess of dPEG* (6.00 g, 1.2 mmol) was dissolved in water for injection (40 mL) and sterile-filtered. The dPEG* solution was then added to the collagen solution, agitated, and the mixture allowed to stand at 17-22°C for about 15 hours. The solution was then centrifuged, and the resulting pellet of

-50-

reconstituted fibrils collected and washed with PBS (3X 400 mL) to remove residual dPEG*. The pellet was then placed in a syringe fitted with a Luer lock hub connected to a second syringe, and was passed between the syringes
5 until homogeneous. The resulting material is a microgel or a particulate suspension of random size fibrils in solution (microgel conjugate). The material is a smooth, pliable, rubbery mass, with a shiny appearance.

(D) Preparation of Cartilaginoid Conjugates:

10 Approximately 20% by weight of dPEG* (pH 7) was added to collagen in solution (33.8 mg/mL), and incubated at 21°C for about 16 hours. The resulting conjugate was washed with 100 mL PBS 3-5 times over 12 hours. The
15 resulting cartilaginoid non-fibrillar collagen-polymer conjugate (NFC-FC cartilage) was a translucent solid with coherent elasticity. The product contained approximately 20-80% fibrillar collagen.

Another NFC cartilage composition was prepared by mixing dPEG* solution (0.6 g, pH 3) with collagen in
20 solution (33.8 mg/mL, pH 2). The mixture was passed between two syringes joined by a Luer lock connector to form a homogenous solution. A solution of dPEG* (20% w/v) in a neutralizing buffer was then added to result in a substantially non-fibrillar collagen (NFC) cartilage
25 material. The resulting product contained approximately 1-40% fibrillar collagen.

Alternatively, fibrillar collagen may be used instead of CIS to produce a cartilaginoid fibrillar collagen-polymer conjugate (FC cartilage) having an
30 opaque appearance and high fibrillar content. Such FC cartilage is more porous and permeable than non-fibrillar biocompatible conjugates.

-51-

Example 2

(Characterization)

(A) Collagen-mPEG prepared in Example 1A was characterized and compared with Zyderm® Collagen Implant (ZCI) and glutaraldehyde-crosslinked fibrillar collagen (GAX).

Extrusion:

This assay measured the force required to extrude the test composition through a 30 gauge needle. The results can be graphed to show that force required (in Newtons) versus plunger travel allowed for a smooth extrusion of ZCI, requiring a force of about 20-30 Newtons. However, if they are graphed as regards GAX, it will show GAX was not extruded smoothly, as shown by the "spiking" exhibited in the force trace. During certain parts of the extrusion, GAX required about 10-15 N for extrusion. In contrast, collagen-mPEG demonstrated a very low extrusion force (8-10 N), with little or no spiking.

Intrusion:

Intrusion is a measure of the tendency of a composition to "finger" or channel into a porous bed, rather than remaining in a compact mass. Low intrusion is preferred in augmentation of soft tissue, so that the injected implant does not diffuse through the dermis and remains in place.

A 1 mL syringe fitted with a 30 gauge needle was half-filled with silicon carbide particles (60 mesh), simulating human dermis. The upper half of the syringe was filled with 0.5 mL test composition (GAX, ZCI, or collagen-mPEG) at 35 mg/mL. The plunger was then fitted, and depressed. On depression, ZCI appeared at the needle, demonstrating intrusion through the silicon

-52-

carbide bed. Syringes filled with GAX or collagen-mPEG of the invention did not pass collagen, instead releasing only buffer, demonstrating no intrudability.

5 Helicity:

 The portion of each composition exhibiting nonhelical character was measured using sensitivity to digestion with trypsin. Samples were treated with the protease trypsin, which is capable of attacking only
10 fragmented portions of the collagen protein. The extent of hydrolysis is measured by fluorescamine assay for solubilized peptides, and the results are expressed as percentage non-helical collagen. The percentage of non-helical collagen was measured 30 minutes after the
15 beginning of the digestion period. The results indicated that ZCI was 3-10% sensitive, GAX was 1-2% sensitive, and collagen-mPEG was about 1% sensitive. Sensitivity to trypsin may also correlate to sensitivity to endogenous proteases following implantation.

20

Collagenase Sensitivity:

 The sensitivity of each composition to collagenase was also measured. ZCI was 65.2% digested, compared to 2.2% for GAX, and 45.8% for collagen-mPEG.

25

Phase Transition:

 The behavior of each composition vs. temperature was examined using a differential scanning calorimeter. On heating, ZCI exhibited multiple peaks at about 45 and
30 53°C. GAX exhibited a peak at 67-70°C. Collagen-mPEG exhibited a peak at 56-61°C.

Lysine Content:

 The number of free lysines per mole was determined
35 for each composition using TNBS to quantify reactive

-53-

epsilon amino groups. ZCI exhibited about 30 lysines per (single helix) molecule (K/m), whereas GAX exhibited 26-27 K/m, and collagen-mPEG 21-26 K/m.

5 (B) Characterization of Crosslinked Biocompatible Conjugates:

10 A collagen-dPEG conjugate prepared as described in Example 1C was characterized using differential scanning calorimetry (DSC). This test is a measure of the transition temperature during fragmentation of the collagen molecule at a microscopic level. A lowering of the transition temperature indicates an increase in fragmentation in a manner similar to that measured by trypsin sensitivity.

15 The collagen-dPEG conjugate showed a single denaturational transition at 56°C by DSC, which is similar to the typical melting point of the collagen-PEG conjugate prepared in Example 1A. By comparison, ZCI has a melting temperature of 45-53°C, with multiple denaturational transitions, and GAX has a melting temperature of 67-70°C, with a single denaturational transition.

20 The extrusion test described in Example 2A could not be used to characterize the collagen-dPEG conjugate because the material was not extrudable through a 30 gauge needle.

25 Using the intrusion test described in Example 2A, the passage of collagen-dPEG was completely blocked at the silicon carbide bed, which indicates high crosslinking between the collagen molecules and little or 30 no intrudability.

- 54 -

Example 3

(Immunogenicity)

(A) Non-crosslinked PEG-Collagen:

This experiment was conducted to demonstrate the relative immunogenicity of a collagen-mPEG preparation of the invention versus a commercially available bovine collagen formulation prepared from essentially the same source material, and having a similar consistency. As both collagen preparations were prepared using atelopeptide collagen (which is only weakly immunogenic), the preparations were formulated with either complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA), to enhance the immune response. This is a severe test, designed to magnify any possible immune reaction.

Collagen-mPEG was prepared as in Example 1A above. Male Hartley guinea pigs (11) were anesthetized and bled by heart puncture for pre-immunization serologic evaluation. Five animals were treated with two 0.1 mL intramuscular injections of Zyderm® Collagen Implant (ZCI) emulsified in CFA (1:9) in the left and right thighs. Another five animals were treated in the same fashion, using collagen-PEG (35 mg/mL) emulsified in CFA. One animal was treated with collagen-PEG in IFA. At day 14 following immunization, all animals were again bled by heart puncture, and serum obtained for antibody titer determination (using ELISA). Serology was again performed at day 30.

On day 30, following collection of serum samples, each animal was challenged intradermally with both ZCI and collagen-PEG (0.1 mL of each, one on each flank). Delayed-type hypersensitivity (DTH) was quantified as a measure of cell-mediated immunity. DTH was evaluated at 24, 48, and 72 hours post-challenge by measuring the diameter of any wheal using micrometer calipers, and noting the extent of erythema and induration. Animals

-55-

were then euthanized with CO₂ and the injection sites excised and fixed in neutral, buffered formalin for histological study.

Serological results indicated reduced immunogenicity of collagen-PEG vs. ZCI. At day 14, 80% of ZCI immunized animals exhibited "positive" antibody responses (titer \geq 160 at day 14), whereas 0% of the collagen-PEG immunized animals exhibited positive responses. At day 30, all ZCI-immunized animals exhibited high antibody titers, whereas none of the collagen-PEG-immunized animals (C-PEG) exhibited high titers. The data are shown in Table 1.

Table 1: Immunogenicity

Animal	Treatment	Antibody Titer	
		day 14	day 30
1	ZCI	320	>2560
2	ZCI	320	1280
3	ZCI	2560	>2560
4	ZCI	320	>2560
5	ZCI	80	2560
6	C-PEG	0	0
7	C-PEG	0	160
8	C-PEG	40	640
9	C-PEG	0	20
10	C-PEG	0	640
11	C-PEG (IFA)	0	160

Responses to the DTH challenge also demonstrated that the collagen-mPEG of the invention is less immunogenic. Guinea pigs immunized with ZCI and challenged with ZCI exhibited a wheel measuring 1.128 ± 0.058 cm in diameter. Animals immunized with collagen-mPEG and challenged with collagen-mPEG exhibited wheals measuring 0.768 ± 0.036 cm. Animals immunized with ZCI and challenged with collagen-mPEG, or immunized with collagen-mPEG and challenged with ZCI, developed wheals smaller than the ZCI-immunized, ZCI-challenged wheals.

-56-

Responses measured at 48 and 72 hours were essentially the same or lower than the 24 hour response for each site. Erythema was essentially the same for all animals.

Histological studies showed that both materials exhibited comparable intrusion, fingering into the dermis and subcutaneous space. Sites of intradermal challenge with ZCI in ZCI-immunized animals exhibited the most extensive inflammatory response, including a cellular infiltrate of lymphohistiocytic elements with eosinophils and occasional giant cells. Two of the implant sites demonstrated an erosive inflammation of the overlying epidermis and eschar formation. Sites of intradermal challenge with collagen-mPEG in ZCI-immunized animals exhibited only a moderate associated inflammatory infiltrate, with a marked reduction in acute cells and lymphoid elements. Histiocytes and giant cells were more prevalent, and in some samples lined and colonized the implants heavily. Animals immunized with collagen-mPEG exhibited only slight to moderate reaction, with ZCI challenge sites accompanied by a modest lymphohistiocytic perivascular infiltrate with a few eosinophils and giant cells. Collagen-mPEG challenge sites were typically accompanied by a minimal scattering of lymphoid cells near the associated vasculature.

25

(B) Crosslinked dPEG-Collagen Conjugates:

Collagen-dPEG conjugates were prepared as in Example 1D. The samples were implanted in the dorsal subcutis and as cranial onlays in rats. After implantation for 30 days in the subcutis, NFC cartilage and NFC-FC cartilage materials had a homogeneous microfibrillar structure. Mild colonization by connective tissue cells occurred at the periphery of the NFC-FC cartilage samples, and mild capsule formation was present. No colonization had occurred with the NFC cartilage material and mild capsule

35

-57-

formation was present. FC cartilage had a very fibrous structure with mild but frequently deep colonization by connective tissue cells and sparse numbers of adipocytes. Trace amounts of capsule were present in limited areas of the FC cartilage samples. NFC cartilage materials tended to retain their pre-implantation shape, with sharply defined edges, while the NFC-FC cartilage samples tended to flatten over time and develop rounded profiles.

When implanted as cranial onlays, the appearance of each of the materials was similar to that in the subcutis except that the samples tended to become anchored to the skull via integration of the capsule or surrounding loose connective tissue with the periosteum.

All of the samples appeared to be biocompatible, have differing degrees of colonization by host tissues, and varying mechanical characteristics.

Example 4

(In situ Crosslinking)

A dPEG solution was prepared as described in Example 1C above. The following samples were then prepared:

- (1) 5 mg dPEG in 80 μ L water, mixed with 0.5 mL fibrillar collagen (35 mg/mL), to a final dPEG concentration of 1% by volume;
- (2) 15 mg dPEG in 80 μ L water, mixed with 0.5 mL fibrillar collagen (35 mg/mL), to a final dPEG concentration of 3% by volume;
- (3) Vitrogen® 100 collagen in solution;
- (4) 5 mg dPEG in 80 μ L water, mixed with 0.5 mL non-fibrillar collagen (35 mg/mL), to a final dPEG concentration of 1% by volume;
- (5) 15 mg dPEG in 80 μ L water, mixed with 0.5 mL non-fibrillar collagen (35 mg/mL), to a final dPEG concentration of 3% by volume;

-58-

- (6) 5 mg dPEG in 0.5 ml PBS, to a final dPEG concentration of 1% by volume; and
- (7) GAX.

5 The dPEG solutions of Samples 1, 2, 4, and 5 were placed in a 1 mL syringe equipped with a Luer lock fitting and connector, and joined to another syringe containing the collagen material. The solutions were mixed by passing the liquids back and forth between the
10 syringes several times to form the homogeneous reaction mixture.

 The syringe connector was then removed and replaced with a 27 gauge needle, and approximately 50 μ L of the reaction mixture was injected intradermally into each of
15 20 guinea pigs. Samples 3, 6, and 7 were similarly administered through a 27 gauge needle. At intervals up to 30 days following injection, the treatment sites were harvested and studied histologically.

 By 30 days, all of the materials appeared to be
20 biocompatible. Samples 1 and 2 displayed wide dispersion with an intermediate degree of interdigitation with dermal collagen fibers. Colonization by connective tissue cells was moderate, and a trace of round cell infiltrate with eosinophils was seen.

25 Samples 3, 4, and 5 were highly dispersed and finely interdigitated with dermal collagen fibers. Colonization was mild to moderate, and trace levels of round cell infiltration were seen.

 Sample 6 had no detectable effects. Sample 7
30 occurred as large islands with moderate colonization and trace to mild levels of inflammation.

-59-

Example 5

(Coating of Implants)

A collagen-dPEG reaction mixture was prepared as described in Example 1C above. A titanium implant was
5 dipped into the reaction mixture approximately 20 minutes after crosslinking was initiated. The implant was then allowed to finish crosslinking and dried overnight.

Example 6

10 (Collagen-Polymer-Growth Factor Conjugates)

(A) A conjugate containing crosslinked collagen-dPEG-TGF- β 1 was prepared as follows:

A solution of TGF- β 1 and ^{125}I -TGF- β 1 (10^5 cpm; 25 μL of 1 mg/mL) was added to a solution of dPEG* (4 mg) in
15 CH_2Cl_2 (100 μL), and the mixture allowed to react for 12 (sample #3) or 35 (sample #5) minutes at 17°C . To this was added 2.5 mL of collagen solution (3 mg/mL atelopeptide nonfibrillar collagen), and the resulting mixture allowed to incubate overnight at ambient
20 temperature. The pellet which formed was collected by centrifugation to provide collagen-dPEG-TGF- β 1.

(B) A composition based on fibrillar atelopeptide collagen was prepared as in part A above, but limiting TGF- β 1/dPEG* reaction time to 2 minutes, and substituting
25 7 mg of fibrillar collagen (precipitated from collagen in solution within 2 minutes prior to use) for collagen in solution.

(C) A composition containing dPEG-crosslinked collagen and free TGF- β 1 was prepared as follows:

30 A solution of dPEG* (4 mg) in CH_2Cl_2 (100 μL) was added to 2.5 mL of CIS (3 mg/mL atelopeptide nonfibrillar collagen), and the resulting mixture allowed to incubate overnight at ambient temperature. The pellet which formed was washed to remove unreacted dPEG*, and 25 μg of
35 TGF- β 1 mixed in to provide collagen-dPEG + TGF- β 1.

- 60 -

(D) The degree of TGF- β 1 binding was determined as follows:

Each composition prepared in parts A-C above was washed six times with 0.5 mL of buffer (0.02 M phosphate buffer, 0.1% BSA) by vigorous, vortexing followed by centrifugation in order to remove non-bound TGF- β 1. The pellet and supernatants were collected at each time of washing, and were counted. The results can be graphed to demonstrate that the TGF- β 1 in the simple mixture is quantitatively released within about 6 washings, while approximately 40% of the TGF- β 1 is retained in the compositions of part B and 50% is retained in the compositions of part A.

(E) The biological activity of the materials prepared above was assayed as follows:

Compositions prepared according to part A (CIS-dPEG-TGF- β 1) (TGF- β 1/dPEG* reaction time of 12 minutes) and part C (CIS-dPEG + TGF- β 1) were prepared, as well as a control prepared according to part C without TGF- β 1 (CIS-dPEG). The samples were washed in PBS/BSA eight times as described in part D, then washed an additional three times in fetal bovine serum (Gibco) at 37°C. This washing protocol resulted in visually detectable material loss, so remaining TGF- β 1 content was determined by counting the remaining ^{125}I . TGF- β 1 activity was then assayed by ELISA. The results are shown in Table 2 below.

30

35

-61-

Table 2: Retention of Biological Activity

Sample	125 _I	remaining	O.D.
	Counts	TGF- β 1 (μ g)	(414 nm)
CIS-dPEG	0	0	0.015 0.015
CIS-dPEG + TGF- β 1	2775	0.5-1.0	0.029 0.035
CIS-dPEG-TGF- β 1	42604	7.4	0.102 0.082

The data demonstrate that the TGF- β 1 retained in the compositions of the invention remains in a substantially active form.

Example 7

(Formulations)

(A) A formulation suitable for implantation by injection was prepared by suspending collagen-PEG in sterile water for injection, at 35 mg/mL. The characteristics of the resulting formulation are described in Example 2 above.

(B) A formulation useful for repair of stress-bearing bone defects (e.g., fractures, nonunions, and the like) may be prepared by mixing collagen-PEG of the invention with a suitable particulate, insoluble component. The insoluble component may be fibrillar crosslinked collagen, gelatin beads, polytetrafluoroethylene beads, silicone rubber beads, hydrogel beads, silicon carbide beads, mineral beads, or glass beads, and is preferably a calcium mineral, for example hydroxyapatite and/or tricalcium phosphate.

Solid formulations were prepared by mixing Zyderm® II Collagen (65 mg/mL collagen) or collagen-mPEG (63

-62-

mg/mL) with particulate hydroxyapatite and tricalcium phosphate (HA+TCP) and air drying to form a solid block containing 65% HA by weight. Optionally, blocks were heat-treated by heating at 75°C for 10 hours. The
 5 resulting blocks were hydrated in 0.13 M saline for 12 hours prior to testing.

On standing, it was observed that Zyderm®-HA+TCP (Z-HA) compositions separated into three phases, whereas PEG-collagen-HA+TCP (PC-HA) compositions remained single
 10 phase.

Each block was elongated by 5%, and its stress relaxation monitored for 1 minute after release. After this test, each block was subjected to constant elongation at a constant 1 cm/min until failure. The
 15 results are shown in Table 3:

Table 3: Mechanical Strength

Sample	<u>Stress Relaxation</u>			<u>Constant Extension</u>	
	Peak Force	Constant Force	t _{1/2} (min)	Rupture Force	Extension at Rupture
Z-HA (air)	1.5	1.1	0.04	2.6	11.0%
	-	-	-	2.6	15.3%
Z-HA (heat)	1.5	1.1	0.06	-	-
	1.4	1.0	0.07	3.4	14.0%
PC-HA (air)	2.6	1.8	0.06	5.5	12.3%
	2.8	2.1	0.08	5.4	11.7%
PC-HA (heat)	3.3	2.6	0.04	5.4	12.0%
	3.6	2.7	0.06	5.4	20.3%

20 All forces reported in newtons. Extension at rupture (strain) reported in percent extension.
 30

The data demonstrate that collagen-polymer forms HA+TCP compositions exhibiting substantially greater tensile strength. Thus, one can prepare implant compositions
 35 with collagen-polymer which are substantially stronger

-63-

than compositions employing the same amount of non-conjugated collagen, or may reduce the amount of collagen-polymer employed to form a composition of equal strength.

5

Example 8

(Crosslinking of Hyaluronic Acid With Difunctional SC-PEG)

One (1) gram of sodium hyaluronate (obtained from LifeCore Biomedical) was added to 15 ml of PBS and allowed to dissolve overnight to form a homogeneous solution. Five (5) ml of the hyaluronic acid/PBS solution was mixed with 50 mg of difunctional SC-PEG in 0.5 ml of PBS using syringe-to-syringe mixing.

15 The resulting material was extruded from the syringe into a petri dish and incubated at 37°C for 16 hours. The material was then allowed to cool at room temperature for 8 hours. After 24 hours, the material had formed a crosslinked gel.

20 Hyaluronic acid without S-PEG was used as a control in this experiment. After the same incubation period, the control was still liquid and runny.

Example 9

(Preparation of Smooth Collagen-Polymer Tube)

25 The needle end was snipped off of a standard 4.5 mm inner diameter syringe containing Zyderm® I Collagen (35 mg/ml, available from Collagen Corporation, Palo Alto, California). Using the syringe plunger, the collagen was pushed out of the cut syringe in a solid cylinder. The collagen cylinder was placed in a petri dish and immersed in a 10% solution of difunctional S-PEG (1.0 g of difunctional S-PEG in 10 ml of PBS).

30 The collagen cylinder was allowed to incubate in the 10% S-PEG solution at room temperature. The crosslinking

-64-

reaction occurs as the PEG diffuses from the outside towards the inside of the collagen cylinder. After 20 - 30 minutes of incubation in the S-PEG solution, the outside of the collagen cylinder had been crosslinked, while the inside remained non-crosslinked.

After 20 - 30 minutes of incubation, the collagen cylinder was removed from the crosslinker solution. The inner, non-crosslinked collagen could easily be squeezed out from the outer crosslinked shell using manual pressure, leaving a hollow tube of PEG-crosslinked collagen.

The hollow tube was then returned to the 10% S-PEG solution and incubated overnight at 37°C in order to complete the crosslinking process.

The outer diameter of the hollow PEG-collagen tube can be varied by varying the size of the collagen cylinder starting material. The inner diameter of the tube can be increased by decreasing the length of time for the initial incubation of the collagen cylinder in the PEG solution. Conversely, the inner diameter of the tube can be made smaller by increasing the initial incubation period.

Example 10

(Preparation of Pleated Collagen-Polymer Tube)

A smooth collagen-polymer tube was prepared according to the method described in Example 9. While still wet, the tube was slipped over the plunger of the same syringe that had originally contained the Zyderm® I Collagen starting material. The tube fit snugly over the syringe plunger. The PEG-collagen tube was then pushed down along the axis of the syringe plunger, forming pleats or ribs in the wet tubing, so that the pleated tube was now approximately half the length of the original smooth tube.

-65-

While still on the syringe plunger, the pleated PEG-collagen tube was dried under the fume hood at room temperature. After 24 hours, the dried pleated tube was pushed off the syringe plunger. The tube retained its pleated shape after removal from the syringe plunger.

The pleated PEG-collagen tube was then placed in a petri dish containing water. The tube retained its pleated shape following rehydration.

10

Example 11

(Preparation of Small Diameter Pleated
Collagen-Polymer Tubing)

0.9 cc of Zyderm® I Collagen was mixed with 0.1 cc of a 5% solution of difunctional S-PEG (5 mg of S-PEG in 0.1 cc of PBS) using syringe-to-syringe mixing. Immediately following mixing, the PEG-collagen material was extruded using an 18-gauge needle into TFE tubing (1.5 mm outer diameter, 1.3 mm inner diameter). (It was necessary to add a certain amount of PEG to provide a starting material with greater structural integrity than straight Zyderm® I Collagen in order to maintain the shape of the small-diameter cylinder.)

After 20 - 30 minutes of incubation at room temperature, the tubing was sliced open and the solid cylinder of PEG-collagen was peeled out of the tubing. The PEG-collagen cylinder was then placed in a petri dish containing 5 cc of a 10% solution of difunctional S-PEG. The crosslinking reaction occurs as the PEG diffuses from the outside towards the inside of the collagen cylinder. After 3 hours of incubation in the S-PEG solution at room temperature, the inside of the cylinder was pushed out using a 1 mm diameter mandrel, resulting in a hollow, smooth PEG-collagen tube.

The PEG-collagen tube was then pushed down along the axis of the mandrel, forming pleats or ribs in the wet

-66-

tubing, so that the pleated tube was now approximately half the length of the original smooth tube.

While still on the mandrel, the pleated PEG-collagen tube was dried under the fume hood at room temperature.

5 After 24 hours, the dried pleated tube was pushed off the mandrel. The tube retained its pleated shape after removal from the mandrel.

10 The pleated PEG-collagen tube was then placed in a petri dish containing water. The tube retained its pleated shape following rehydration.

PEG-collagen tubes of different diameters can be prepared by using different sizes of TFE tubing and varying the time for the crosslinking reaction to occur.

15

Example 12

(Preparation of Thin-Walled Tubes)

0.90 ml of Zyderm® I Collagen was mixed with a solution of 10 mg of difunctional S-PEG in 0.10 ml of PBS using syringe-to-syringe mixing.

20

A TFE tube having an inner diameter of 0.9 mm was placed inside another TFE tube having an inner diameter of 1.2 mm. The PEG-collagen mixture was injected through a 27-gauge needle into the space between the inner and outer tubes. The tubing was then incubated at 37°C for 2 hours.

25

The outer tubing was pulled off and the inner tubing with the PEG-collagen shell around it was incubated at 37°C for an additional 2 hours.

30

The thin PEG-collagen shell was then carefully pushed off of the inner TFE tubing. The resulting PEG-collagen tube was clear and cellophane-like in consistency.

35 The PEG-collagen tube was then placed in water to rehydrate. Although the tube was very thin and had a small diameter, water could be injected through it.

-67-

The thickness of the tube wall and the inner diameter of the collagen-polymer tube can be varied by varying the size of the inner and outer TFE tubes used to mold the collagen-polymer material. Thin-walled tubes produced according to the method described above may be especially suited for use as nerve guide tubes to facilitate nerve regeneration.

Example 13

10 (Preparation of PEG-Collagen Strings)

Five (5) ml of Zyderm® I Collagen (35 mg/ml) was mixed with 50 mg of difunctional SG-PEG in 0.5 ml of phosphate buffered saline (PBS) using syringe-to-syringe mixing. The material was immediately transferred to either 1.5 or 3.5 mm diameter Teflon® tubing, then incubated at 37°C for 16 hours. The crosslinked collagen gels were removed from the tubing and dried overnight. The strings were held taut during drying to ensure that the strings would dry in the radial, rather than axial, dimension.

Non-crosslinked collagen strings were produced as a control by mixing 5 ml of Zyderm® I Collagen with 0.5 ml of PBS. The method described above was used to produce strings of two different diameters.

25 Diameter, length, and weight of the strings were measured in the fresh (wet), dehydrated, and rehydrated states. Results of these measurements are presented graphically in Figure 1 and in the table shown in figure 14.

30 Because the non-crosslinked strings do not contain the hydrophilic PEG, they were not able to take up water and rehydrate. Therefore, no measurements were obtained for these strings in the rehydrated state. The strings retained all of their original length and nearly all of their original diameter and weight upon rehydration.

-68-

Various rheological measurements were performed on the crosslinked and non-crosslinked strings in their dehydrated states. All strings were cut to a constant length of 20 mm prior to rheological evaluation. Results are presented in the table shown in figure 15. A bar graph showing the swellability of strings is shown in Figure 1. Bar graphs with error bars showing standard deviation for several rheological measurements on each of the four string types are presented in Figures 2-5.

10 Tensile stress (N/mm^2) is a measure of the force at failure (breakage) of the string as a function of its cross-sectional area. Strain ($\Delta \text{length}/\text{length}$) and Δ length are measures of the elasticity of the string (how much it will stretch under tension). Young's Modulus
15 (N/mm^2) is calculated by dividing stress by strain and is known as the rheological "fingerprint" of a particular material.

The bar graphs in Figures 2-5 illustrate the large standard deviation and variability of the rheological
20 measurements obtained for the non-crosslinked strings, showing the non-homogeneity of the non-crosslinked materials. The consistent results obtained with the PEG-crosslinked strings show that PEG crosslinking imparts
homogeneity, as well as greater mechanical strength and
25 elasticity, to the collagen material.

Example 14

(Preparation of Coiled Strings)

A small-diameter crosslinked collagen string was
30 produced as described in Example 13 by injecting collagen in a 1% solution of difunctional S-PEG through an 18-gauge needle into TFE tubing (0.9 mm outer diameter, 0.6 mm inner diameter).

Following removal from the tubing, the wet string
35 was coiled around a second piece of TFE tubing having an

-69-

outer diameter of 1.5 mm. The coiled string was dried on the tubing for 2 days at room temperature under the fume hood.

5 The dehydrated PEG-collagen coil was pushed off the tubing. The coiled string in its dried state was manually pulled straight. The now-straight string was immersed in water and quickly returned to its coiled shape upon rehydration.

10 The coiled wet string was removed from the water bath, pulled and dried straight under tension. The straight dried string was again immersed in water and again returned to its original coiled shape upon rehydration.

15 The collagen-polymer coils can be pulled straight to facilitate delivery through a needle or catheter. They are especially useful in the treatment of aneurysms because of their ability to rehydrate to the coil shape and expand to fill the void.

20 The above example illustrates the "memory" of the collagen-polymer material. Upon rehydration, the material returns to the original shape in which it was first dried.

Example 15

25 (Rheological Properties of PEG-Collagen Materials)

A 10% solution of activated difunctional SG-PEG was prepared by diluting 100 mg of powdered difunctional SG-PEG (3400 dalton MW) in 1 ml of phosphate buffered saline (PBS). One (1) ml of the 10% difunctional SG-PEG
30 solution was mixed with 9 ml of Zyderm®I Collagen (Z-I, 35 mg/ml) to achieve a final PEG concentration of 1%. The collagen and crosslinker solution were placed in 10-ml syringes and mixed using syringe-to-syringe mixing.

35

-70-

Zyderm®II Collagen (Z-II, 65 mg/ml) was crosslinked with difunctional SG-PEG using the same method described above.

5 The syringes containing the Z-I-PEG and Z-II-PEG composites were incubated at 37°C for 16 hours and formed polymerized gels.

10 The needle end of each of the two syringes was cut off and the gels pushed out of the barrels of the syringes using the respective syringe plunger. The solid gels were then sliced into disks of 2 mm thickness, dehydrated, and then rehydrated. Diameter, thickness and weight of the disks were measured in the fresh (wet), dehydrated, and rehydrated states. Results of these measurements are presented in the table shown in figure 16.

15 The crosslinked collagen disks (at both collagen concentrations) regained nearly all of their original dimensions upon rehydration.

20

Example 16

(Coating of Platinum Wires)

Lidocaine-free Zyderm® II Collagen (65 mg/ml, available from Collagen Corporation, Palo Alto, CA) was diluted to 32.5 mg/ml using sterile-filtered phosphate buffered saline (PBS). A 10% solution of activated difunctional S-PEG was prepared by diluting powdered difunctional S-PEG in sterile-filtered PBS. One hundred (100) µl of the S-PEG was added to 900 µl of the collagen to achieve a final S-PEG concentration of 1%. The S-PEG solution and collagen were placed in 3-ml syringes and mixed using syringe-to-syringe mixing.

30 Platinum wires (#1, available from Target Therapeutics, Santa Clara, CA) coiled to a diameter of 0.25 mm on mandrels (inner wires to keep coils straight) were placed inside ultra micro pipet tips (0.5 - 10 µl),

-71-

one coil per tip, so that the mandrel passed through both the narrow and wide openings of the pipet tip. The S-PEG - collagen mixture in the syringe was injected into each of the pipet tips using a 22-gauge needle.

5 The pipet tips containing the coils were placed in a petri dish and allowed to incubate at 37°C for twenty-four hours. The tips containing the coils were placed under the laminar flow hood at room temperature for six days. The coated coils were then removed from the pipet
10 tips. The coatings will not adhere well to the coils if drying time is not adequate.

The coated coils can be delivered using a Tracker® 325i catheter (Target Therapeutics) or other similar type catheter.

15

Example 17

(Coating of Platinum Wires)

Lidocaine-free Zyderm® II Collagen was diluted to 32.5 mg/ml using sterile-filtered PBS. A 10% solution of
20 activated difunctional S-PEG was prepared by diluting powdered difunctional S-PEG in sterile-filtered PBS. One hundred (100) µl of the S-PEG solution was added to 900 µl of the collagen to achieve a final S-PEG concentration of 1%. The S-PEG solution and collagen
25 were placed in 3-ml syringes and mixed using syringe-to-syringe mixing.

Coiled platinum wires on mandrels were placed inside 1-mm diameter Teflon® tubing. The S-PEG - collagen mixture in the syringe was injected into the tubing over
30 the coils using a 22-gauge needle. Plugs were inserted into the ends of the tubing and the tubing was placed on a rack to hold the end of the coils straight in the center of the tubing. The rack was placed inside a 37°C incubator for sixteen hours.

35

- 72 -

After removal from the incubator, the tubing was cut laterally with a scalpel and peeled away from the coils. The coated coils were allowed to air dry at room temperature for several hours, then removed from the
5 mandrels.

The coated coils can be delivered via catheter.

The invention is shown and described herein at what is considered to be the most practical, and preferred
10 embodiments. It is recognized, however, that departures may be made therefrom which are within the scope of the invention and that obvious modifications will occur to one skilled in the art upon reading this disclosure.

15

Example 18

(Preparation of Telopeptide-containing Collagen-Polymer Conjugates)

Telopeptide-containing collagen and atelopeptide collagen were crosslinked using difunctionally activated
20 SG-PEG (MW = 3400 dalton). Physical properties of the resulting materials were compared.

Preparation of PEG Crosslinked Telopeptide-containing Collagen

25 Nine hundred (900) ml of cow hide slurry (sampled just prior to pepsin digestion) was stabilized to 17°C in a water batch for one hour. One hundred (100) ml of 0.2M phosphate buffer, pH 11.4, was added to the hide slurry with rapid stirring. After incubating for 16 hours at
30 17°C, the material was centrifuged to produce a 91 gram pellet of telopeptide-containing collagen. The protein concentration of the pellet was determined to be 40 mg/ml using the Biuret assay.

A 3-ml sample of the pellet was acidified by adding
35 0.5 ml of 0.1 M hydrochloric acid (HCl). The resulting

-73-

material was very opaque and fibrillar at acidic pH (4 - 5). One-half (0.5) ml of the acidified telopeptide-containing collagen was placed in a mold and 0.25 ml of a 35.7% (wt.%/vol.%) concentration difunctionally activated SG-PEG solution was added. The mold containing the collagen and crosslinker solution was incubated overnight at 37°C. Crosslinking occurred as the PEG solution diffused into the collagen gel. Five strips (30 mm x 10 mm x 2 mm thickness) of tightly crosslinked telopeptide-containing collagen gel were obtained.

Preparation of PEG Crosslinked Atelopeptide Collagen

Zyderm® II Collagen (65 mg/ml concentration; available from Collagen Corporation, Palo Alto, CA) was diluted to a concentration of 40 mg/ml using phosphate buffered saline (PBS). Three (3) ml of the resulting 40 mg/ml atelopeptide collagen was acidified and crosslinked with difunctionally activated SG-PEG according to the method described above. Five strips (30 mm x 10 mm x 2 mm thickness) of tightly crosslinked atelopeptide collagen were obtained.

Fibril Formation

Three (3) ml of telopeptide-containing collagen pellet and 3 ml of Zyderm II Collagen were acidified as described above. One-half (0.5) ml of each of the two acidified collagen materials were placed in molds. The molds containing the acidified telopeptide-containing and atelopeptide collagen were incubated at 37°C overnight.

After incubation at 37°C overnight, a moderately crosslinked telopeptide-containing collagen gel was obtained. The atelopeptide collagen failed to produce any crosslinked gel.

-74-

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) is used to measure the melting temperature of a material. The DSC measurements for non-crosslinked and PEG-crosslinked
 5 telopeptide-containing collagen and atelopeptide collagen are presented in Table 4 shown below and Figures 6 and 7.

Table 4. DSC Measurements

	<u>Material</u>	<u>Peak(s)</u>	<u>T_m(°C)</u>
10	Atelopeptide collagen	Doublet	45, 55
	Telopeptide-containing collagen	Doublet	52, 57
15	Crosslinked atelopeptide collagen	Singlet (Sharp)	59
	Crosslinked telopeptide-containing collagen	Broad	66

20 Non-crosslinked atelopeptide collagen shows two temperature transition peaks, one at approximately 46°C and a second at approximately 54°C. Non-crosslinked
 25 telopeptide-containing collagen also has two temperature transitions, one occurring at 49°C and the second occurring at 57°C. The peaks for the telopeptide-containing collagen are broader than those for the atelopeptide collagen because the telopeptide-containing collagen contains indigenous crosslinks that are removed
 30 during enzyme treatment and are not present in the resulting purified atelopeptide collagen.

When atelopeptide collagen is crosslinked with difunctionally activated S-PEG, the transition temperature is increased to approximately 58°C and
 35 appears as a single peak. When telopeptide-containing

-75-

collagen is crosslinked with difunctionally activated S-PEG, the transition temperature is increased to approximately 66°C and consists of a single transition peak which is somewhat broader than the comparable transition peak for the PEG-crosslinked atelopeptide collagen. The broad peak obtained for the crosslinked telopeptide-containing collagen is likely due to increased heterogeneity as a result of the indigenous crosslinks, as compared with the crosslinked atelopeptide collagen, which is more homogeneous as a result of enzyme treatment and purification.

Rheological Properties

Non-crosslinked and PEG-crosslinked telopeptide-containing collagen and atelopeptide collagen were tested for tensile strength (N), tensile stress (N/mm²), strain ($\Delta L/L$), and Young's Modulus (N/mm²). Results of the rheological testing are presented in the table shown in figure 17.

Tensile stress (N/mm²) is a measure of the force at failure (breakage) of a material as a function of its cross-sectional area. Strain ($\Delta \text{length}/\text{length}$) is a measure of the elasticity of a material (how much it will stretch under tension). Young's Modulus (N/mm²) is calculated by dividing stress by strain and is known as the rheological "fingerprint" of a particular material.

As shown by the data in the table of figure 17, the crosslinked telopeptide-containing collagen is significantly stronger than the crosslinked atelopeptide material.

Swellability

Non-crosslinked and PEG-crosslinked telopeptide-containing and atelopeptide collagen were tested for swellability. The data are presented in Table 5.

-76-

Table 5. Swellability Data

	<u>Material</u>	<u>Original Wet Weight (g)</u>	<u>Dehydrated Weight (g)</u>	<u>Rehydrated Weight (g)</u>
5	Atelo	0.175	0.009	---
	Telo	0.325	0.027	0.207
	Atelo-PEG	0.166	0.020	0.084
	Telo-PEG	0.398	0.026	0.409

Because the non-crosslinked atelopeptide collagen is not a gel, it fell apart when immersed in water and, therefore, no rehydrated weight value could be obtained.

As shown by the data, PEG-crosslinked telopeptide-containing collagen shows even better rehydration properties than crosslinked atelopeptide collagen, regaining 100% of its original wet weight after rehydration.

The instant invention is shown and described herein at what is considered to be the most practical and preferred embodiments. It is recognized, however, that departures may be made therefrom which are within the scope of the invention and that obvious modifications will occur to one skilled in the art upon reading this disclosure.

25

30

35

-77-

WHAT IS CLAIMED:

1. A biocompatible, biologically inert conjugate comprising a natural polymer or derivative thereof
5 chemically conjugated by an ether bond to a synthetic hydrophilic polymer.
2. The conjugate of claim 1, wherein the natural polymer or derivative thereof is selected from the group
10 consisting of a glycosaminoglycan and derivatives thereof.
3. The conjugate of claim 2, wherein the glycosaminoglycan or derivatives thereof is selected from
15 the group consisting of chondroitin sulfate A (4-sulfate), chondroitin sulfate C (6-sulfate) dermatan sulfate (chondroitin sulfate B), hyaluronic acid, and derivatives thereof.
- 20 4. The conjugate of claim 2, wherein the natural polymer is a polysaccharide selected from the group consisting of hyaluronic acid and dextran.
5. The conjugate of claim 4, wherein the
25 polysaccharide is a dextran selected from the group consisting of hydroxyethyl cellulose, cellulose ether, starch, and cyclodextrin.
6. The conjugate of claim 1, wherein the natural
30 polymer is collagen or a derivative thereof.
7. The conjugate of claim 6, wherein the collagen is selected from the group consisting of reconstituted atelopectide fibrillar collagen and telopeptide-
35 containing fibrillar collagen.

-78-

8. The conjugate of claim 1, wherein the synthetic hydrophilic polymer is selected from the group consisting of a difunctionally activated polyethylene glycol and a multifunctionally activated polyethylene glycol.

5

9. A composition, comprising:

a biocompatible, biologically inactive conjugate comprised of a natural polymer or derivative thereof chemically conjugated by an ether bond to a synthetic

10 hydrophilic polymer; and

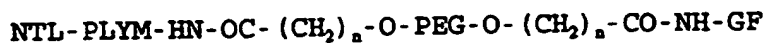
a therapeutically effective amount of a cytokine or growth factor.

10. The composition of claim 9 wherein the cytokine
15 or growth factor is selected from the group consisting of epidermal growth factor, transforming growth factor- α , transforming growth factor- β , transforming growth factor- β 2, platelet-derived growth factor-AA, platelet-derived growth factor-AB, platelet-derived growth factor-BB,
20 acidic fibroblast growth factor, basic fibroblast growth factor, connective tissue activating peptide, β -thromboglobulin, insulin-like growth factors, tumor necrosis factor, interleukins, colony stimulating factors, erythropoietin, nerve growth factor,
25 interferons, bone morphogenic protein and osteogenic factors and

wherein the synthetic hydrophilic polymer is a difunctionally activated polyethylene glycol.

30

11. The composition of claim 9, wherein the conjugate has the following general structural formula:



35

-79-

wherein n is an integer selected from the group consisting of 0, 1, 2, 3, or 4, NTL-PLYM is a natural polymer or derivative thereof, PEG is polyethylene glycol, and GF is a growth factor or cytokine.

5

12. A flowable, injectable, pharmaceutically acceptable composition, comprising:

a biocompatible conjugate comprised of an inert, natural polymer or derivative thereof, chemically
10 conjugated by an ether bond to a synthetic, non-immunogenic hydrophilic polymer; and

a sufficient amount of a fluid, pharmaceutically acceptable carrier to render the composition containing the conjugate flowable and injectable.

15

13. A composition suitable for repair of bone defects, which comprises:

an inert, natural, biologically inactive polymer or derivative thereof, chemically conjugated via an ether
20 linkage to a synthetic hydrophilic polymer;

a suitable particulate material; and

a sufficient amount of a fluid, pharmaceutically acceptable carrier.

25

14. The composition of claim 13 wherein the natural polymer is selected from the group consisting of collagen and a glycosaminoglycan; and

wherein the suitable particulate material is selected from the group consisting of fibrillar,
30 crosslinked, atelopeptide collagen, gelatin beads, polytetrafluoroethylene beads, silicone rubber beads, hydrogel beads, silicon carbide beads, glass beads, hydroxyapatite particles, tricalcium phosphate particles, or mixtures of hydroxyapatite and tricalcium phosphate
35 particles.

-80-

15. The composition of claim 13 wherein said particulate material comprises hydroxyapatite particles, tricalcium phosphate particles, or mixtures of hydroxyapatite and tricalcium phosphate particles having
5 an average diameter of about 20 to 250 microns in diameter.

16. A method for augmenting tissue in a mammal, which comprises:
10 providing an aqueous mixture of a natural polymer or derivative thereof;
providing an aqueous composition of synthetic, non-immunogenic, hydrophilic polymer having a reactive group capable of forming a covalent ether bond in situ with the
15 natural polymer;
mixing the natural polymer mixture with the synthetic polymer composition to form a reaction mixture; and
administering the reaction mixture at a site in need
20 of augmentation before substantial crosslinking occurs between the natural polymer and the synthetic polymer.

17. A string having a diameter in the range of about 0.10 mm to about 20 mm, the string comprising a
25 natural polymer or derivative thereof chemically conjugated to a synthetic non-immunogenic hydrophilic polymer by a covalent bond.

18. The string of claim 17, wherein the string is
30 dehydrated, has a circular cross-section, is in the form of an elongated solid cylinder, and has a diameter in the range of 0.25 mm to about 2.5 mm.

- 81 -

19. The string of claim 18, wherein the string is flexible, and wherein the covalent bond is selected from the group consisting of an ester linkage, a urethane linkage, and an ether linkage.

5

20. A tube having an outer diameter in the range of about 0.25 mm to about 5.0 cm and an inner diameter in the range of 0.05 mm to 4.95 cm, the tube comprising a natural polymer or derivative thereof chemically
10 conjugated to a synthetic non-immunogenic hydrophilic polymer by a covalent bond.

21. The tube of claim 20, wherein the tube is dehydrated.

15

22. The tube of claim 20, wherein the tube has a circular cross-section.

23. The tube of claim 20, wherein the tube has a
20 length of more than about 10 mm.

24. The tube of claim 20, wherein the tube has a length of greater than 10 cm and is flexible;

wherein the covalent bond is selected from the group
25 consisting of an ester linkage, a urethane linkage, and an ether linkage; and

wherein the synthetic hydrophilic polymer is a difunctionally activated polyethylene glycol and the natural polymer is collagen or a derivative thereof.

30

25. A flowable, injectable, pharmaceutically acceptable composition, comprising:

a biocompatible conjugate comprised of a natural polymer or derivative thereof, chemically conjugated to a
35 synthetic, non-immunogenic hydrophilic polymer; and

-82-

a sufficient amount of a fluid, pharmaceutically acceptable carrier to render the composition containing the conjugate flowable and injectable.

5 26. A solid article having coated on the surface the conjugate of any of claims 1-8.

27. The article of claim 26 wherein the article is a bone implant having the porous surface.

10

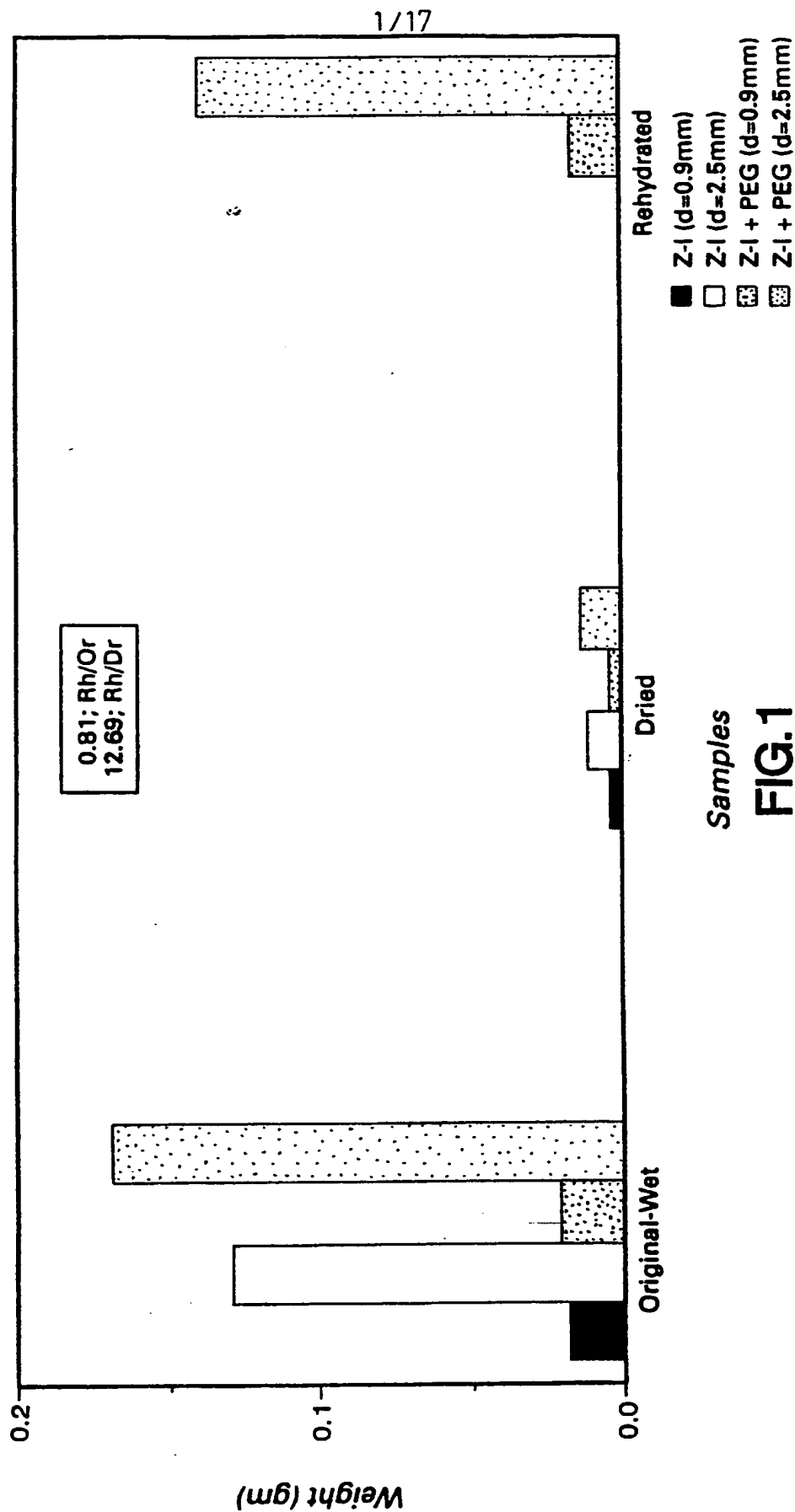
15

20

25

30

35



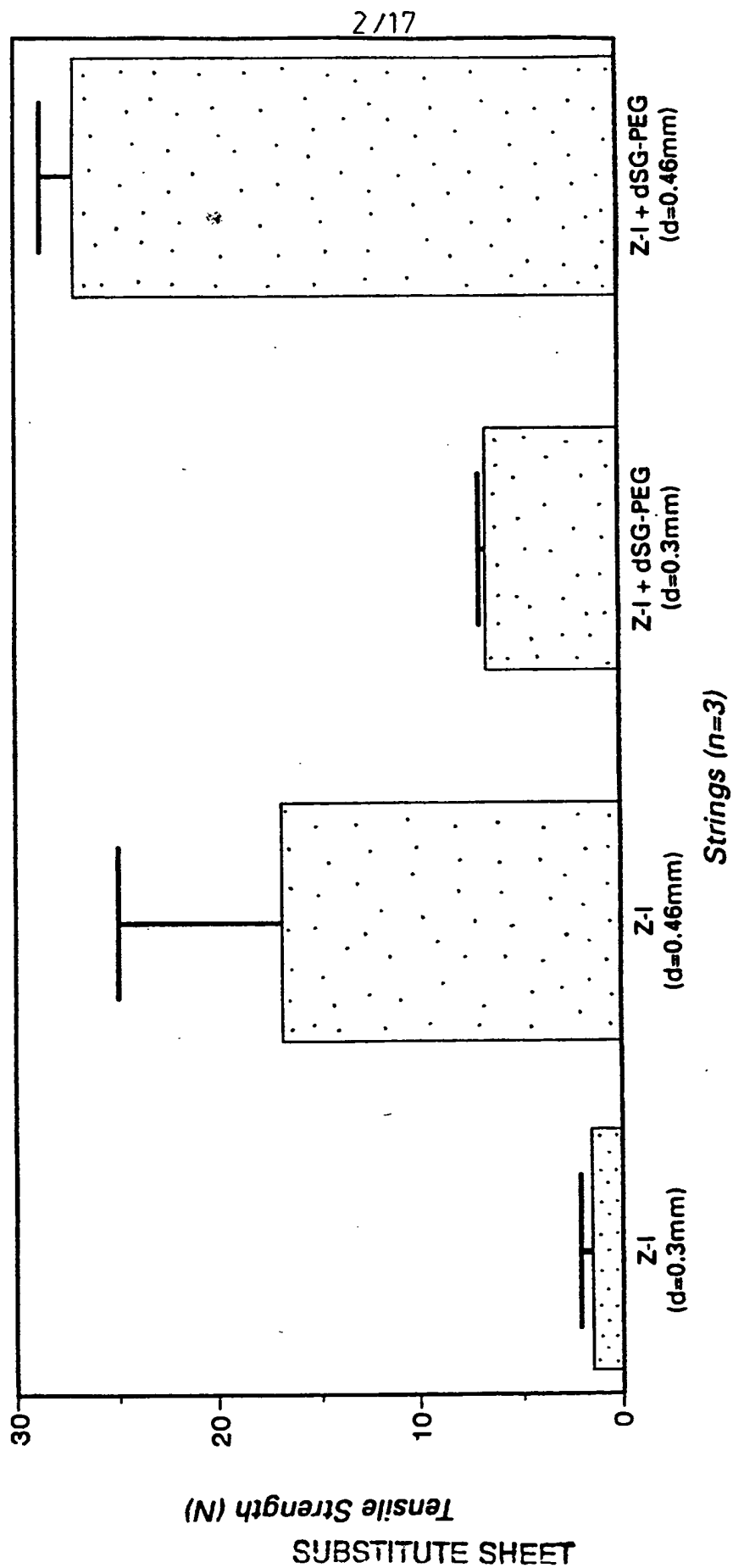
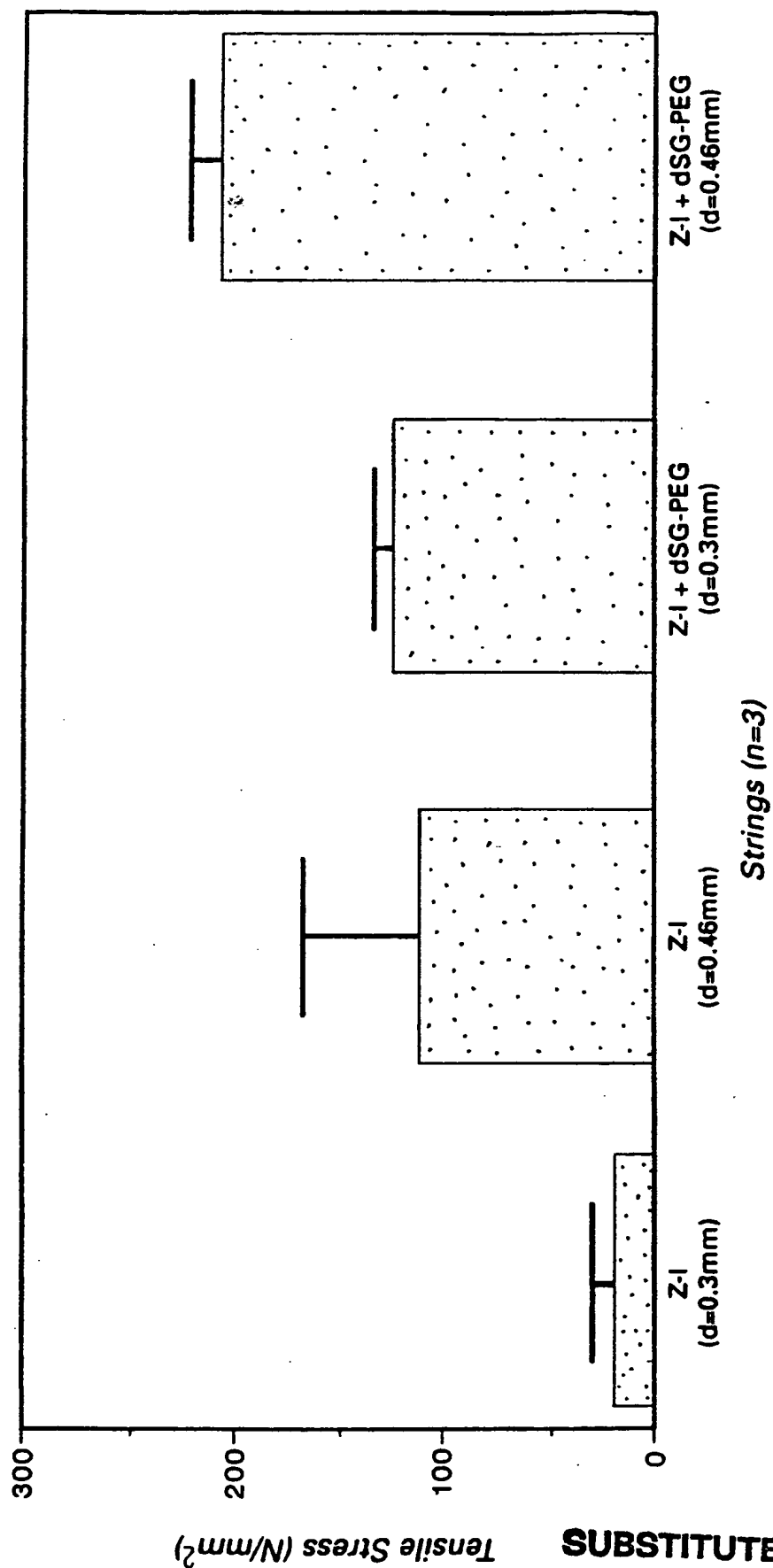


FIG.2



SUBSTITUTE SHEET

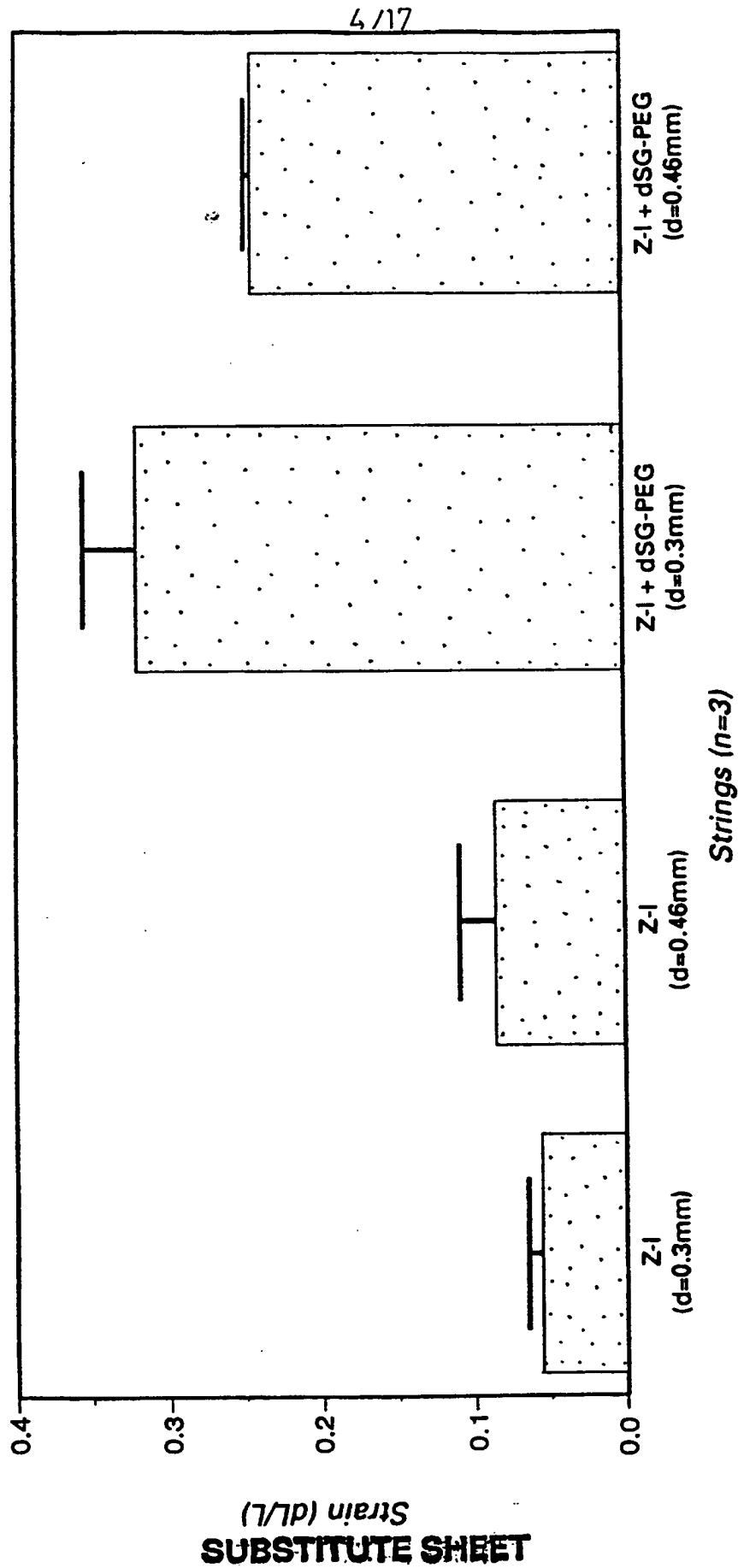


FIG.4

5/17

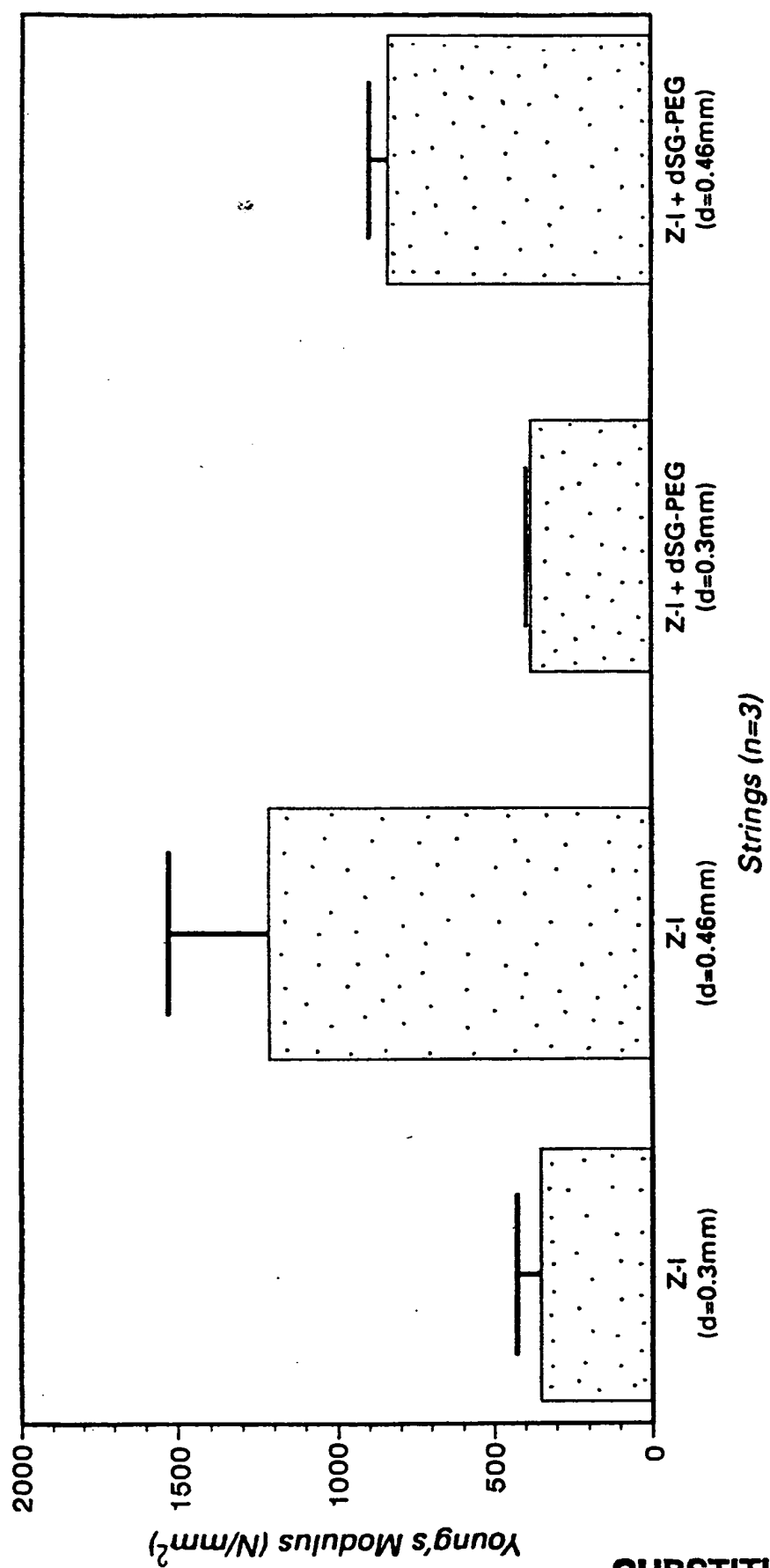


FIG.5

SUBSTITUTE SHEET

6/17

	Atelopeptide Collagen	PEG-Collagen
Weight:	40.10	33.2
Peak Temp:	52.83	58.4

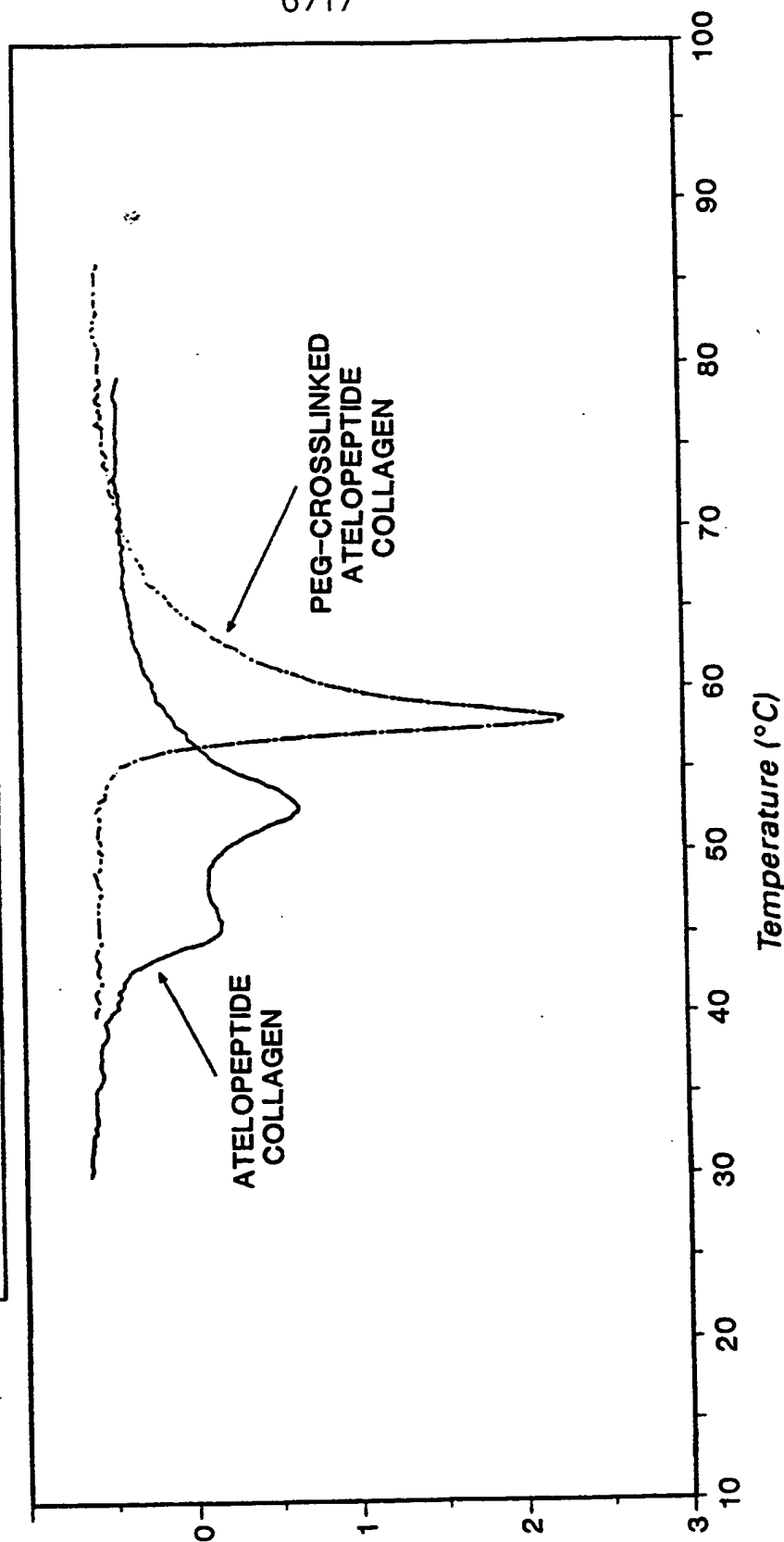


FIG.6

7/17

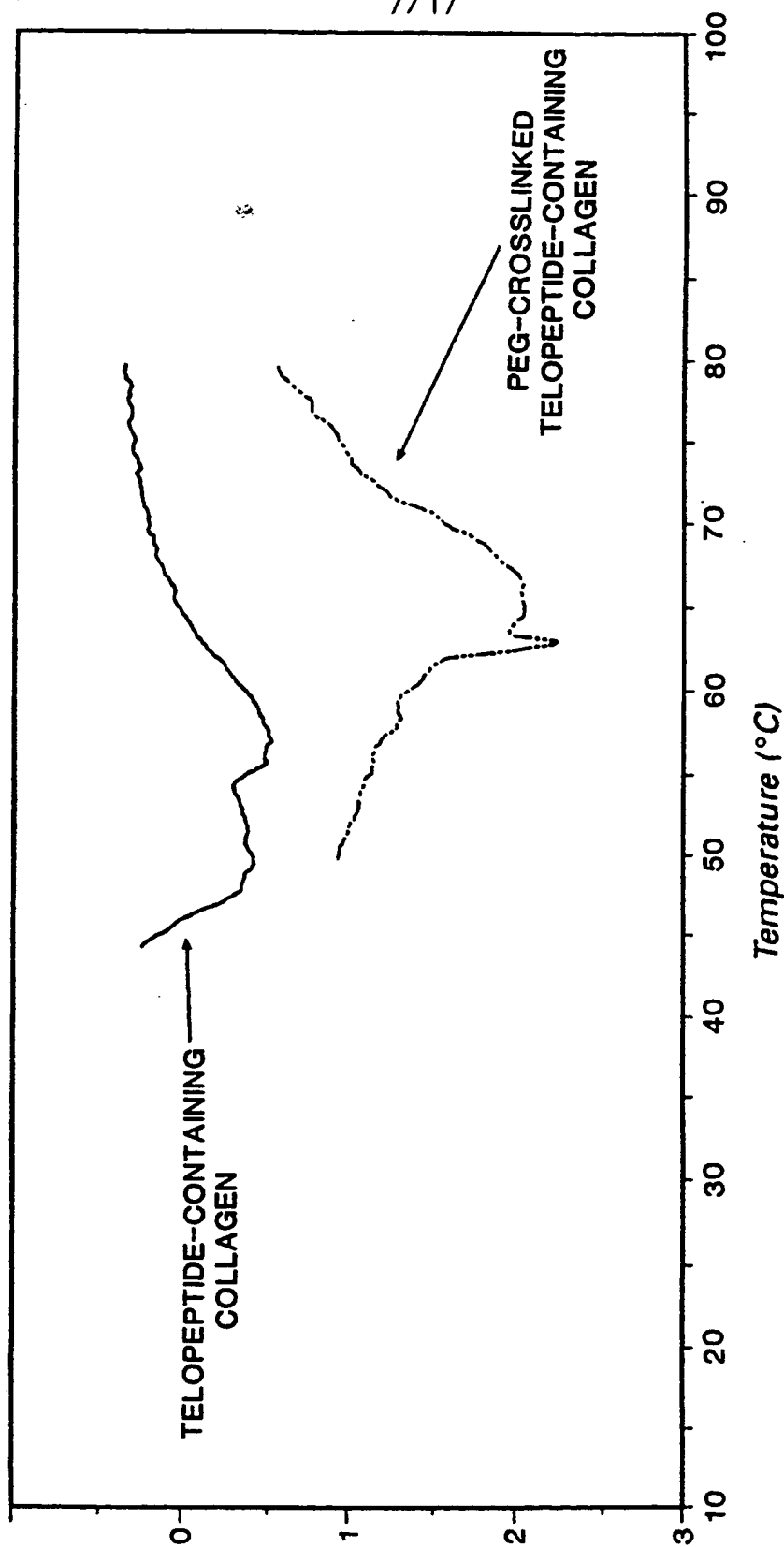


FIG.7

Heat Flow (mW) EXO

↓

8/17

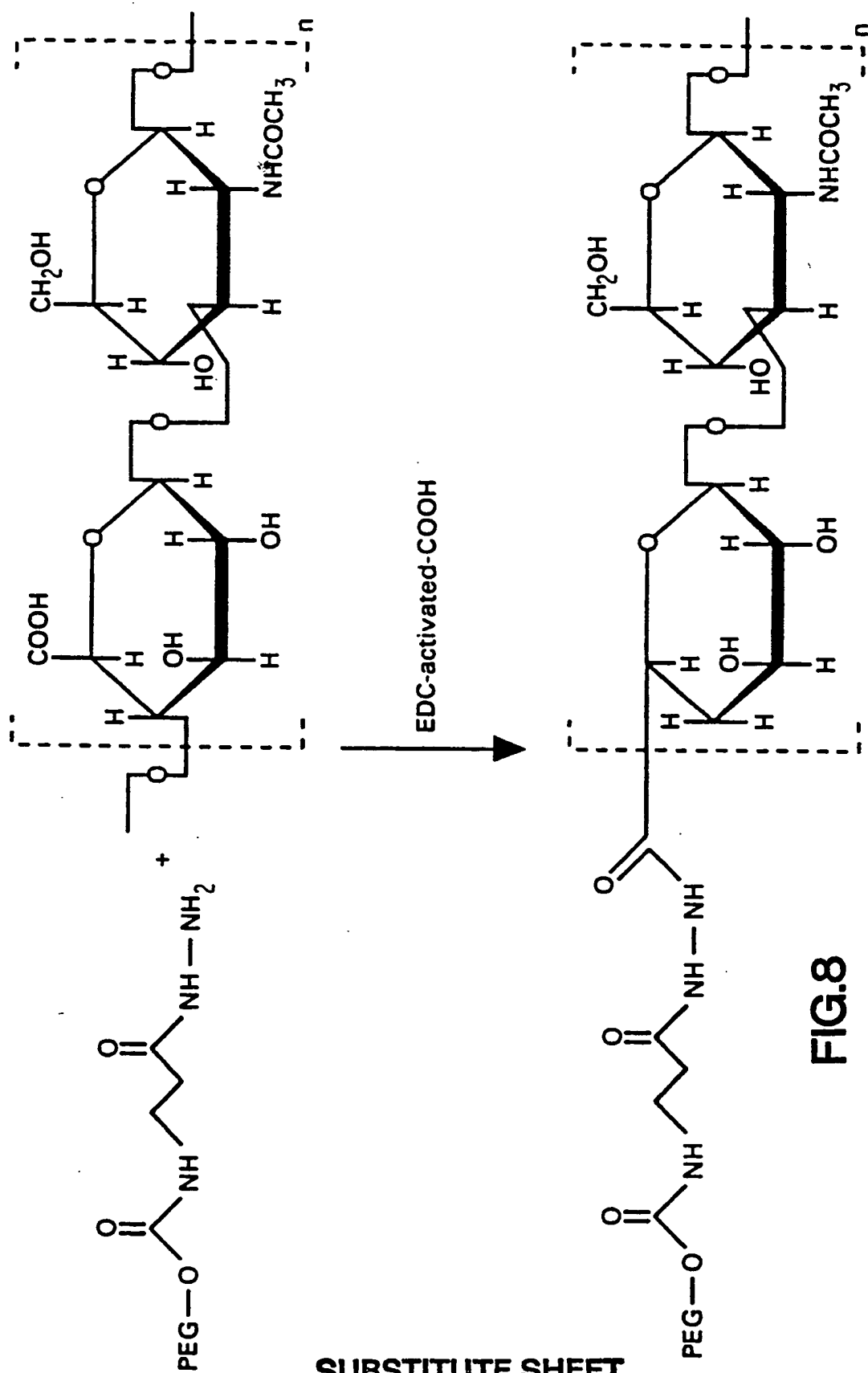


Fig. 8

9/17

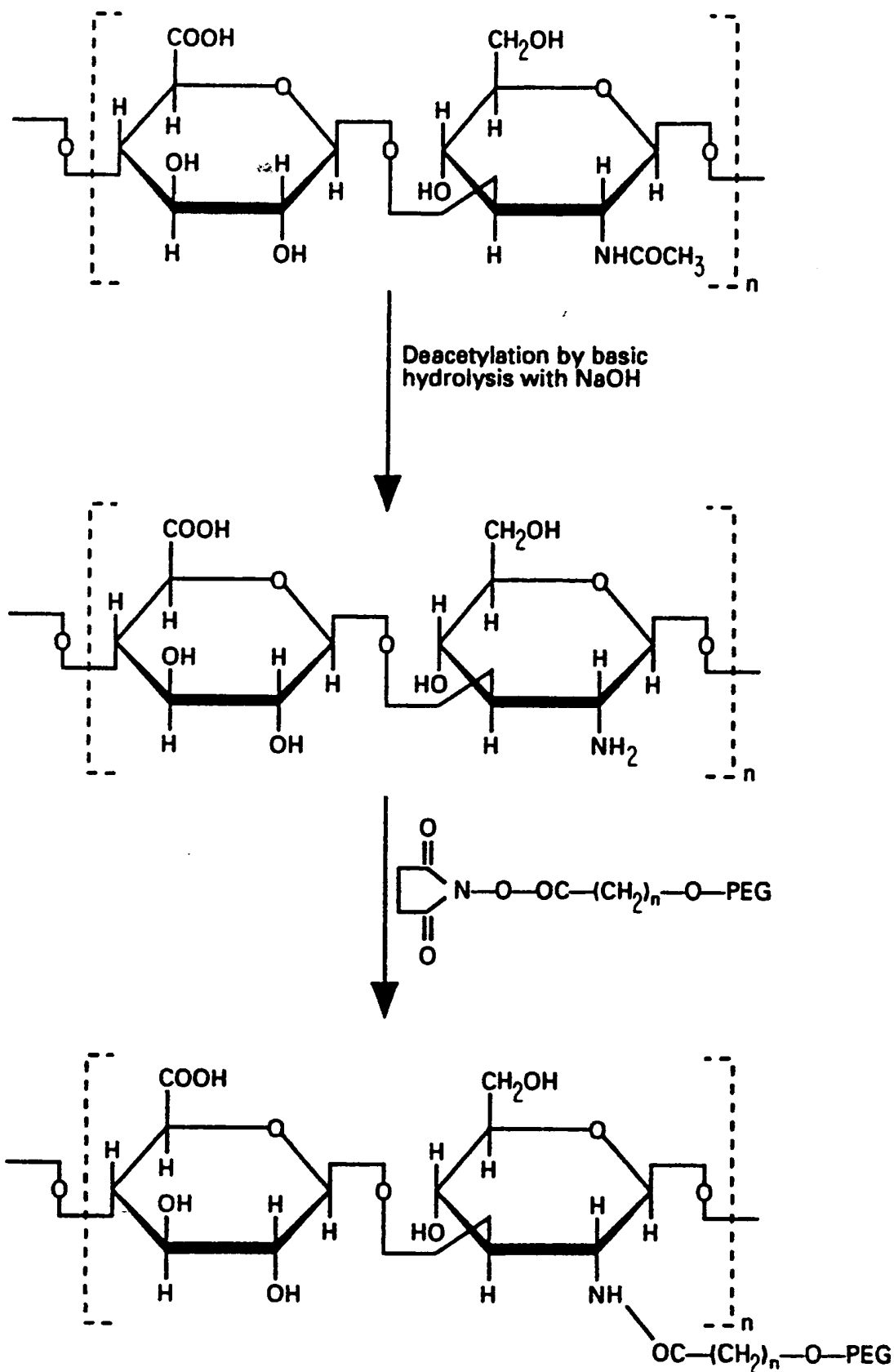


FIG.9

10/17

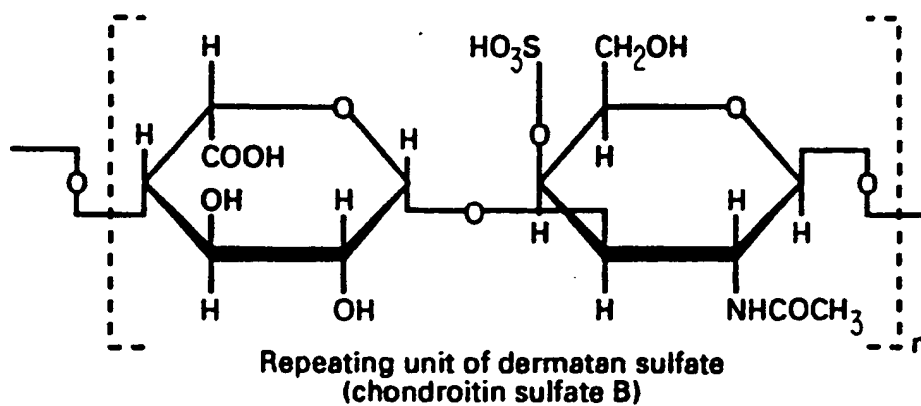
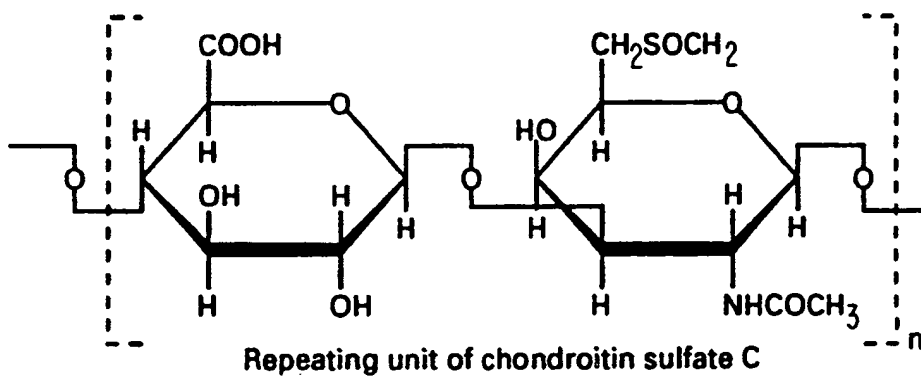
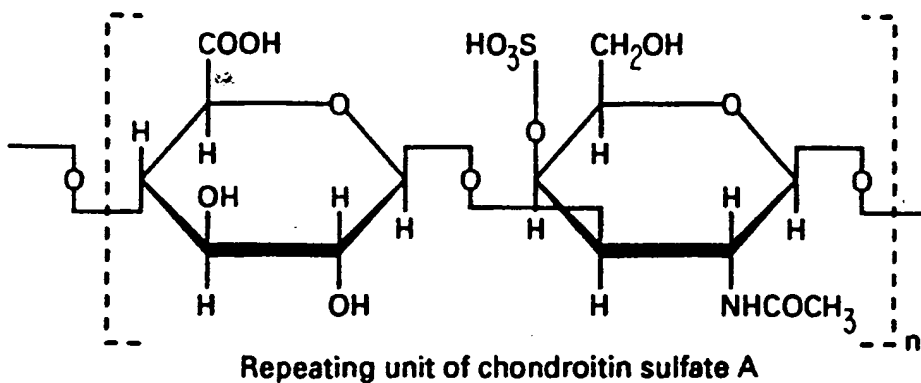


FIG.10

SUBSTITUTE SHEET

11/17

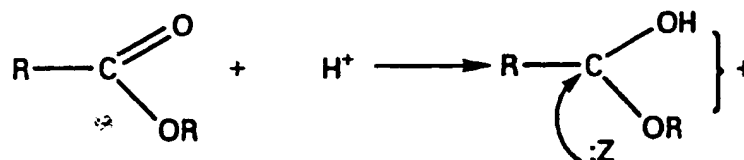


FIG. 11a

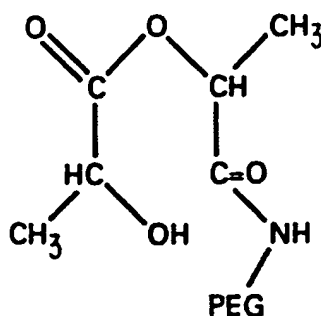
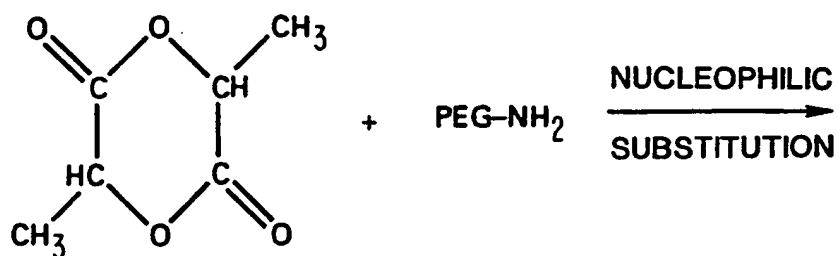


FIG. 11b

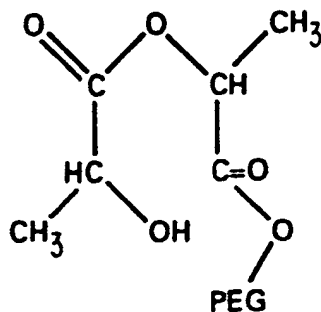
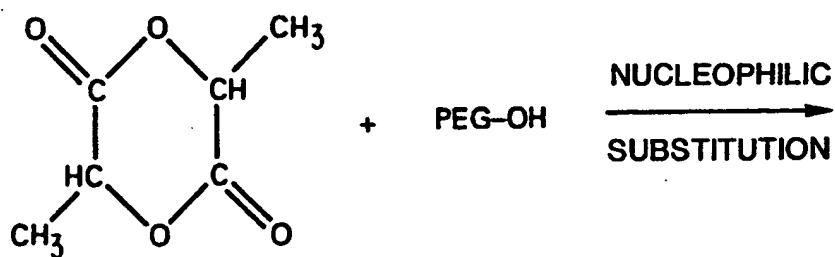
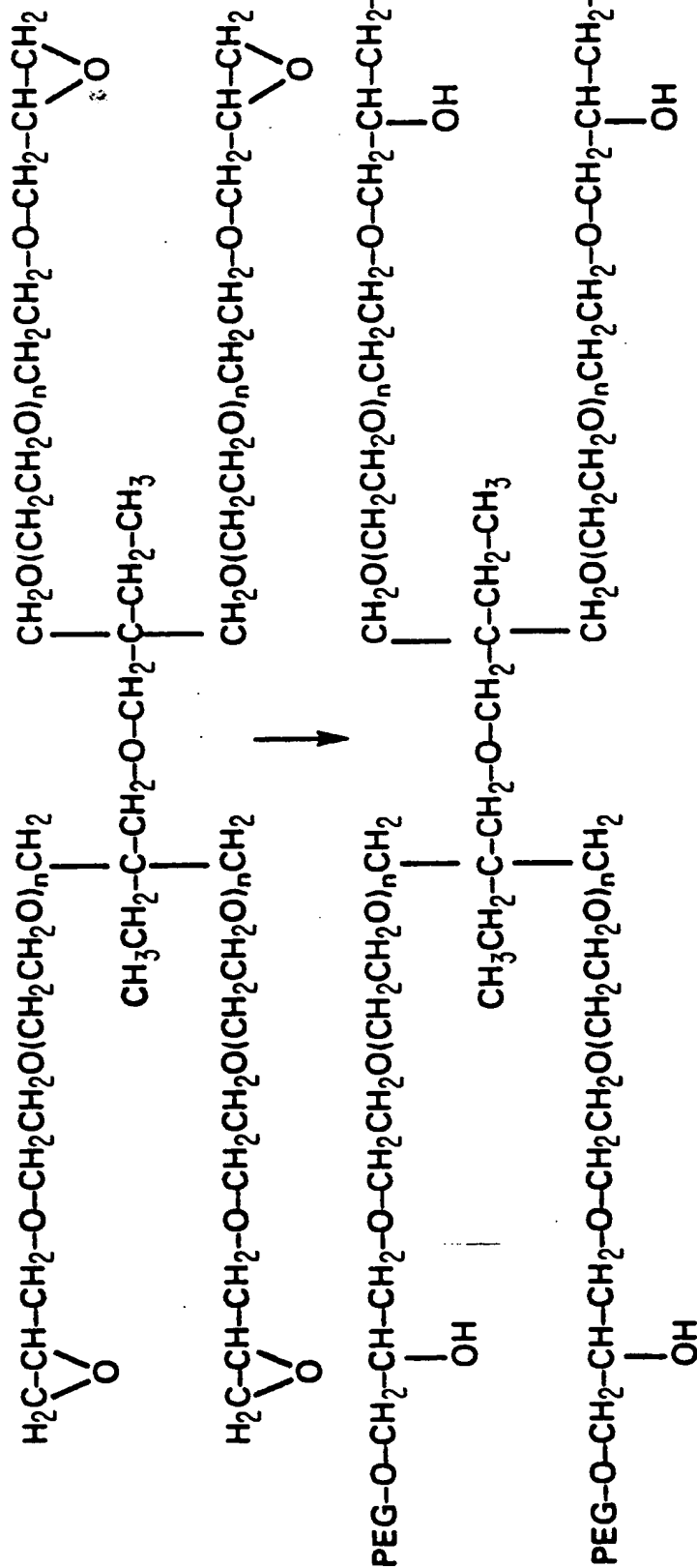


FIG. 12

MULTIFUNCTIONAL E-PEG

4 PEG-OH +

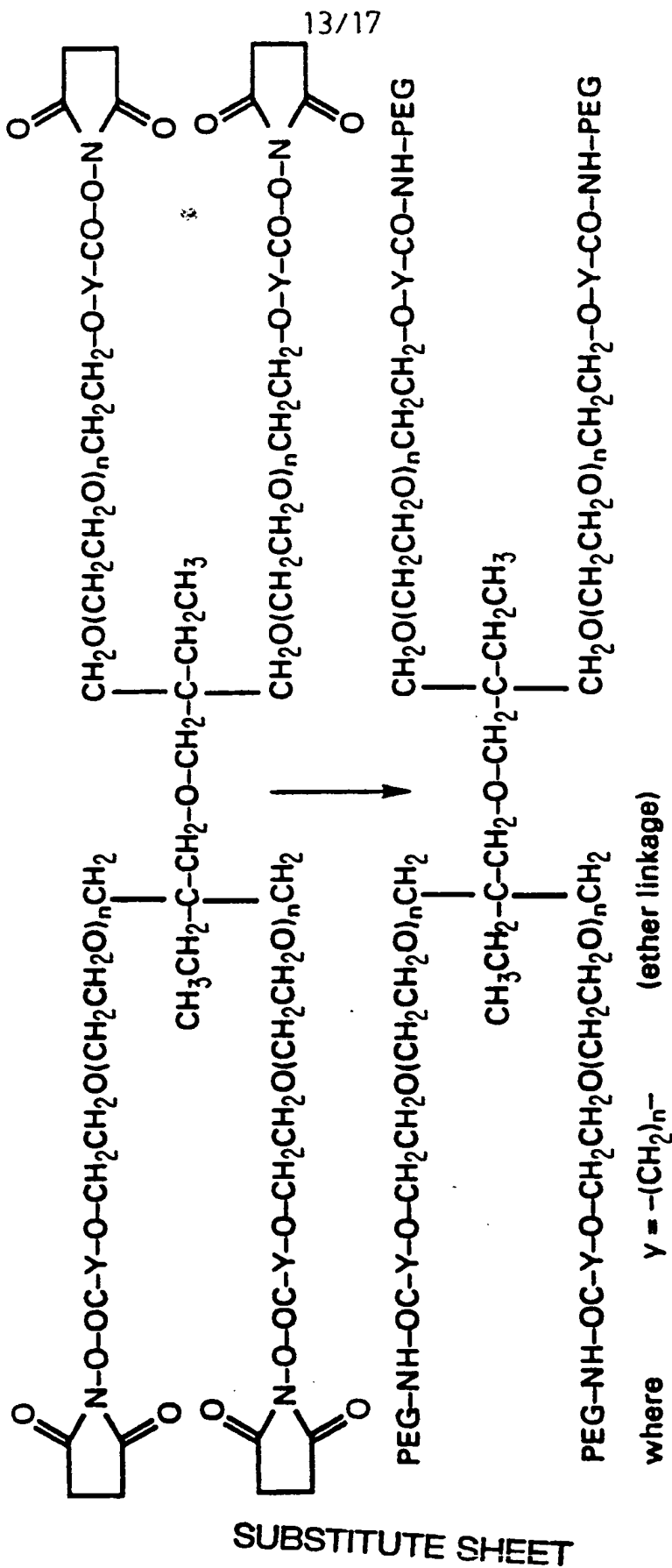
 $n = 0, 1, 2, 3, 4, 5$

12/17

FIG. 13

MULTIFUNCTIONAL S-PEG

4 PEG-NH₂ +



where

$\gamma = -(\text{CH}_2)_n-$ (ether linkage)

○=

or $\gamma = -\text{C}(\text{CH}_2)_n-$ (ester linkage)

$$n = 0, 1, 2, 3, 4, 5$$

15/17

FIG.15

Sample*	Diameter (mm)	Length (mm)	Tensile Strength (N)	Tensile Stress (N/mm ²)	Δ Length (mm)	Strain ($\Delta L/L$)	Young's Modulus (N/mm ²)
Z-I	0.30	20	1.0 2.0 1.0	14.9 30.3 14.9	1.0 1.4 1.0	0.0488 0.0676 0.0488	305 448 305
Z-I+PEG	0.30	20	6.5 6.0 6.5	127 112 131	7.7 6.5 8.5	0.3257 0.2814 0.3541	391 402 370
Z-I	0.46	20	18.2 24.0 8.0	121 160 51	2.0 2.2 1.2	0.0953 0.1040 0.0583	1264 1537 875
Z-I+PEG	0.46	20	29.0 26.0 25.5	223 198 197	5.5 5.4 5.6	0.2429 0.2390 0.2468	916 829 796

* Z-I = Zyderm[®]I Collagen

16/17

FIG.16

sample*	Diameter (mm)			Thickness (mm)			Weight (grams)		
	Fresh	Dehyd.	Rehyd.	Fresh	Dehyd.	Rehyd.	Fresh	Dehyd.	Rehyd.
Z-I+PEG	14	12	13.1	2.0	0.6	1.9	0.3213	0.0215	0.3048
Z-II+PEG	14	10	13.7	2.0	1.0	1.8	0.4111	0.0385	0.3943

* Z-I = Zyderm® I Collagen
 Z-II = Zyderm® II Collagen

17/17

FIG.17

Material	Tensile Strength (N)	S.D.	Tensile Stress (N/mm ²)	S.D.	Strain ($\Delta L/L$)	S.D.	Young's Modulus (N/mm ²)
Atelo (n=3)	0	0	0	0	0	0	0
Telo (n=3)	0.67	0.21	0.051	0.017	0.414	0.020	0.122
Atelo-PEG (n=3)	1.17	0.15	0.072	0.010	0.210	0.009	0.342
Telo-PEG (n=3)	2.00	0.10	0.165	0.018	0.497	0.082	0.335

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/06292

A. CLASSIFICATION OF SUBJECT MATTER IPC(5) : C08G 63/48, 63/91; C08H 1/00; A61K 37/12 US CL : 525/54.1, 937; 523/113, 115; 530/356, 840 According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 525/54.1, 937; 523/113, 115; 530/356, 840 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)														
C. DOCUMENTS CONSIDERED TO BE RELEVANT														
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X	(RAMSHAW) ANALYTICAL BIOCHEMISTRY, Vol. 141, pages 361-365, published 1984 "Precipitation of Collagens by Polyethylene Glycols". Note the Abstract and page 361 under the section titled "Experimental Procedures.	1-3 4-8												
Y	US, A, 4,495,285 (SHIMIZU) 22 JANUARY 1985. Note the Abstract and column 3, line 37 to column 6, line 2.	1, 2, 8												
Y	US, A, 4,496,689 (MITRA) 29 JANUARY 1985. See the Abstract, column 5, line 6 to column 6, line 27, and the paragraph bridging column 7 to column 8.	1, 2, 8												
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.														
<table border="0"> <tr> <td>* Special categories of cited documents:</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be part of particular relevance</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier document published on or after the international filing date</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"G" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"G" document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means		"P" document published prior to the international filing date but later than the priority date claimed	
* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention													
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone													
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art													
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"G" document member of the same patent family													
"O" document referring to an oral disclosure, use, exhibition or other means														
"P" document published prior to the international filing date but later than the priority date claimed														
Date of the actual completion of the international search 13 OCTOBER 1993		Date of mailing of the international search report 02 DEC 1993												
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE		Authorized officer NATHAN M. NUTTER Telephone No. (703) 308-2351												

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06292

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,847,325 (SHADLE) 11 JULY 1989. See the Abstract and column 4, lines 26-55. Also note column 4, line 59 to column 5, line 9 and column 12, lines 17-38.	1, 2, 8
A	US, A, 3,949,073 (DANIELS) 06 APRIL 1976. See the entire document.	1-8
A	US, A, 4,424,208 (WALLACE) 03 JANUARY 1984. See the entire document.	1-8

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I. Claims 1-8 drawn to a conjugate of a natural polymer to a synthetic polymer classified in class 525, subclass 54.1.

Group II. Claims 9-11 drawn to a pharmaceutical composition classified in class 424, subclass 78.17.

Group III. Claims 12 and 25 drawn to an injectable pharmaceutical composition classified in class 514, subclasses vary.

Group IV. Claims 13-15 drawn to a pharmaceutical composition having a particulate material classified in class 424, subclass 489 plus.

Group V. Claim 16 drawn to a method of augmenting tissue classified in class 623, subclass 11 plus.

Group IV. Claims 17-19 drawn to a string classified in class 606, subclass 228.

Group VII. Claims 20-24 drawn to a tube classified in class 623, subclass 12.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER: _____**

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.